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CARNITINE PALMITOYL TRANSFERASE 1 – A POTENTIAL TARGET TO RESTORE DYSREGULATED METABOLISM IN NEURODEGENERATIVE DISEASES?

BASED ON *IN VIVO* MODELS MIMICKING AMYOTROPHIC LATERAL SCLEROSIS, PARKINSON'S DISEASE AND MULTIPLE SCLEROSIS

BY
MICHAEL SLOTH TRABJERG

DISSERTATION SUBMITTED 2020

AALBORG UNIVERSITY
DENMARK

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CURRICULUM VITAE

Michael Sloth Trabjerg, born 1991 in Herning, Denmark.

EDUCATION:

2013 – 2017: MSc in Medicine (MD), Aalborg University Hospital, Denmark

2016: Personal license in Laboratory Animal Science, EU function ABD (obtained at Southern University of Denmark)

2010 – 2013: B.Sc. in Medicine, Aalborg University, Denmark

POSITIONS:

2020 - 2021: Research Assistant, Department of Health Science and Technology, Laboratory of Molecular Pharmacology, Aalborg University, Denmark.

2017 - 2020: Ph.D.-student, Department of Health Science and Technology, Laboratory of Molecular Pharmacology, Aalborg University, Denmark.

External stay: 2019: Stony Brook University, New York. Collaboration with Professor Liliana M. Davalos and Associate Professor Angelique Corthals on 16s rRNA sequencing and fecal microbiota.

2017 – 2017: Research Assistant, Department of Health Science and Technology, Laboratory of Metabolism Modifying Medicine, Aalborg University, Denmark.

2016 – 2019: Substitute Doctor at the Psychiatric Department, Aalborg University Hospital.

2014 – 2017: Student teacher in Clinical Skills, School of Medicine and Health Sciences, Aalborg University, Denmark.

2011 – 2017: Student Assistant, School of Medicine and Health Sciences, Aalborg University. Representing Aalborg University and the Faculty of Medicine at different venues for example education meetings for high school students.

TEACHING AND SUPERVISION:

Teaching experience in anatomy, physiology, pharmacology and pathology at 1st – 6th semester bachelor education in medicine and medicine with industrial

specialization. Supervision of bachelor (3rd, 4th and 6th semester) and master students (9th and 10th semester). Supervision of one medical research year student. Approximately 1600 hours.

GRANTS:

2017: Juchum Foundation 130.000 d.kr.: Parkinson's disease (Principal investigator)

2017: Speciallæge Heinrich Kopps Legat og Stinne og Martinus Sørensen Fond 20.000 d.kr.: Parkinson's disease (Principal investigator)

2017: Gangsted Fonden 400.000 d.kr.: PhD-scholarship within the area of CNS diseases with focus and on Parkinson's disease and Amyotrophic lateral sclerosis (Co-applicant, Principal investigator)

2018: Aage og Johanne Louis-Hansens Fonden 3.000.000 d.kr: PhD-scholarship within the area of mitochondrial dysfunctions role in amyotrophic lateral sclerosis (Co-applicant, Principal investigator)

2019: Svend Andersen Fonden 120.000 d.kr.: Research scholarship to medical student Dennis C. Andersen (Principal investigator)

2019: A.P. Møller Lægefonden 50.000 d.kr.: CPT1A role in Amyotrophic lateral sclerosis (Principal investigator)

2019: Torben og Alice Frimodts Fond 25.000 d.kr.: CPT1 role in PARK2 mouse model (Principal investigator)

2019: The Foundation for Neurological Research 33.995 d.kr: The gut microbiota's role in Parkinson's disease (Principal investigator)

2019: Knud og Edith Eriksens Mindefond 2019 25.000 d.kr.: The role of lipid metabolism in Hd82gln mouse model mimicking Huntington disease (Principal investigator)

2019: Carl og Ellen Hertz's Legat til dansk læge- og naturvidenskab: 10.000 d.kr.: The role of lipid metabolism in *Park2* mouse model of Parkinson's disease (Principal investigator)

2019: Kong Christian Den Tiendes Fond 28.600 d.kr.: Lipid metabolism in an in vivo model of Huntington disease (Principal investigator)

2020: A. P. Møller Lægefonden 55.000 d.kr.: Dysregulated lipid metabolism in C9orf72 mouse model of Amyotrophic lateral sclerosis (Principal investigator)

2020: Torben og Alice Frimodts Fond 15.000 d.kr.: The role of dysregulated lipid metabolism in Lrrk2 mouse model mimicking Parkinson's disease (Principal investigator)

2020: The Foundation for Neurological Research 20.000 d.kr.: The role of dysregulated lipid metabolism in SOD1 G93A mice (Principal investigator)

SCIENTIFIC WORK:

Peer reviewed publications:

1. Identification of brain antigens recognized by autoantibodies in experimental autoimmune encephalomyelitis-induced animals treated with etomoxir or interferon-β. Mørkholt, A. S.; Kastaniegaard, K.; **Trabjerg, M. S.**; Gopalasingam, G.; Niganze, W.; Larsen, A.; Stensballe, A.; Nielsen, S.; Nieland, J. D. (2018). *Scientific Reports*, 8(1).

2. 2. CPT1A plays a key role in the development and treatment of multiple sclerosis and experimental autoimmune encephalomyelitis. Mørkholt, A. S., **Trabjerg, M. S**., Oklinski, M. K. E., Bolther, L., Kroese, L. J., Pritchard, C. E. J., Huijbers, I. J., Nieland, J. D. V. (2019). *Scientific Reports*, *9*(1).

3. 3**.** Dysregulation of metabolic pathways by carnitine palmitoyl-transferase 1 plays a key role in central nervous system disorders: experimental evidence based on animal models. **Trabjerg, M. S**; Mørkholt, A. S.; Lichota J.; Oklinski, M. K. E.; Wiborg, O.; Andersen, D. C.; Jønsson, K.; Mørk, K.; Skjønnemand, M-L. N; Kroese, L. J.; Pritchard, C. E. J.; Huijbers, I. J.; Gazerani, P.; Corthals, A.; Nieland, J. D. V. *Sci Rep* **10,** 15583 (2020)

Submitted publications, in review:

4. Downregulating carnitine palmitoyl transferase 1 affects disease progression in the SOD1 G93A mouse model of ALS. **Trabjerg MS**, Andersen DC, Huntjens P, Oklinski KE, Bolther L, Hald JL, Baisgaard AE, Mørk K, Warming N, Kullab UB, Kroese LJ, Pritchard CEJ, Huijbers IJ, Nieland JDV. [*Submitted*]

5. Downregulation of carnitine palmitoyl transferase 1 is highly efficacious in mouse models mimicking Parkinson's disease. **Trabjerg MS**, Andersen DC, Huntjens P, Mørk K, Warming N, Kullab UB, Skjønnemand M-L N, Oklinski MK, Oklinski KE, Bolther, Kroese LJ, Pritchard CEJ, Huijbers IJ, Corthals A, Nieland JDV. [*Submitted*]

Conference activities:

1. Comparison of etomoxir, a lipid metabolism blocker, and interferon beta treatment on antibody recognition of brain proteins in multiple sclerosis. / Mørkholt, Anne Skøttrup; Kastaniegaard, Kenneth; **Trabjerg, Michael Sloth**; Gopalasingam, Gopana; Niganze, Wanda; Oklinski, Michal Krystian; Larsen, Agnete; Nieland, Jette G. K.; Stensballe, Allan; Nielsen, Søren; Nieland, John Dirk. International Journal of MS Care, Volume 19. Suppl. 1, (NP01), 2017, p. 54.

2. Identifying the Role of Lipid Metabolism in an Experimental Autoimmune Encephalomyelitis Mice Model. / Mørkholt, Anne Skøttrup; **Trabjerg, Michael Sloth**; Huijbers, Ivo; Pritchard, Colin; Kroese, Lona; Nielsen, Søren; Nieland, John D. International Journal of MS Care, Volume 20. Suppl. 1, (NI02), 2018, p. 60.

3. Identifying the Role of Lipid Metabolism in Central Nervous Systems Diseases: Is There a Common Theme for Multiple Sclerosis, Amyotrophic Lateral Sclerosis, Parkinson's Disease, and Depression? / **Trabjerg, Michael Sloth**; Mørkholt, Anne Skøttrup; Nielsen, Søren; Nieland, John D. International Journal of MS Care, Bind 20, Nr. Suppl. 1, (NP01), 2018, p. 65.

4. Blocking Carnitine palmitoyl-transferase 1 (CPT1) potentially delays disease progression in the SOD1 G93A mouse model. / **Trabjerg, Michael Sloth**; Andersen, Dennis Christian; Nieland, John Dirk. European Network to Cure ALS Meeting 2018, Book of Abstracts, June 20 - 22 2018, Oxford, p. 41.
5. Is Lipid Metabolism the Missing Link in Brain Diseases? John D. Nieland, **Michael Trabjerg**, Anne Moerkholt, Michal K. Oklinski, Luise Bolther, Ivo Huijbers, Colin Pritchard, Lona Kroese, Angelique Corthals. International Journal of MS Care, Volume 21, Suppl. 1, 2019, p. 41.

6. The role of lipid metabolism in mouse models of Parkinson's disease. **Michael Sloth Trabjerg**; Dennis Christian Andersen; Pam Huntjens; Kasper Mørk; Marie-Louise Skjønnemand; Michal Krystian Oklinski; Anne Skøttrup Mørkholt; Ivo Huijbers; Colin Pritchard; Lona Kroese; John Dirk Nieland. Movement Disorders. Volume 34. Suppl. 2. 734. The International Parkinson and Movement Disorder Society Meeting 2019, Nice, France.

Oral presentations:

1. Is There a Difference for the Role of Lipid Metabolism in Multiple Sclerosis, Parkinson's Disease and Amyotrophic Lateral Sclerosis? / **Trabjerg, Michael Sloth**. Annual Meeting, Consortium of Multiple Sclerosis Centers, June 2, 2018, Nashville.

2. The role of dysregulated lipid metabolism in amyotrophic lateral sclerosis. Research meeting at Department of Neurology, Aalborg University Hospital. February 2019.

3. The role of lipid metabolism and carnitine palmitoyl transferase 1 in Amyotrophic lateral sclerosis and Parkinson's disease. / **Trabjerg, Michael Sloth**. Mini symposium on the role of metabolism and diet on the development of brain diseases. Department of Health Science and Technology, Aalborg University. April 25th 2019, Aalborg, Denmark.

ENGLISH SUMMARY

In the last century, neurological disorders have been recognized as individual entities and categorized by their pathological characteristics' such as motor neuron disease, neurodegenerative or demyelinating. However, in the last decades, it has become clear that they share several common pathogenic mechanisms, and that most central nervous system (CNS) diseases have disruption of homeostasis in multiple systems outside the CNS. The neurodegenerative diseases amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and multiple sclerosis (MS) are all characterized by inflammation, oxidative stress, mitochondrial dysfunction, dysregulated hypothalamic-pituitary-adrenal axis and pathological changes in the gut microbiota. Additionally, in ALS, PD and MS the metabolism is dysregulated, characterized by a shift from the metabolism of glucose to the metabolism of lipids.

The CNS utilises glucose as its primary fuel but under pathological conditions the metabolism can shift towards lipid metabolism. Lipid metabolism promotes a variety of pathological processes such as inflammation, oxidative stress and mitochondrial dysfunction. The gate-keeper of the metabolism of lipids is carnitine palmitoyl transferase 1 (CPT1), which is located at the outer mitochondrial membrane and facilitates the transport of long-chain fatty acids into the mitochondrial matrix. In the mitochondrial matrix, fatty acids undergo β-oxidation to form acetyl-CoA that subsequently can generate ATP through the Krebs cycle and electron transport chain. However, the acetyl-CoA from the β-oxidation exerts negative feedback to glucose metabolism through downregulation of the pyruvate dehydrogenase complex, which establishes a vicious cycle promoting lipid metabolism and thereby mitochondrial dysfunction, oxidative stress and inflammation.

In addition, the Inuit, a population living in the northern part of Canada and Greenland, have a high prevalence of the proline for leucine at codon 479 (P479L) mutation in the *CPT1A* gene. This results in a downregulation of the CPT1A activity to 22% compared to the wild type (wt) protein. Interestingly, these people have a remarkably low prevalence of disease like MS, depression and possibly also ALS compared to their background population. This could indicate a potentially protective mechanism towards neurodegeneration by the downregulation of CPT1A activity.

Based on this, the effects of pharmacological downregulation of CPT1 activity and genetic downregulation of CPT1A activity by *Cpt1a* P479L mutation were evaluated in animal models mimicking ALS (SOD1 G93A model), PD (rotenone and *Park2* mutation model) and MS (experimental induced encephalomyelitis, EAE). We hypothesised that downregulation of CPT1 lipid metabolism would result in delayed disease progression or resistance to disease induction due to amelioration of disease mechanisms such as inflammation, oxidative stress and mitochondrial dysfunction. In addition, the effects of stimulating lipid metabolism by environmental stressors were

evaluated in the SOD1 G93A (diet and corticosterone) and EAE (diet) models. This was hypothesized to exacerbate disease progression and pathogenic mechanisms. The findings are presented in the following five manuscripts:

Manuscript I presents data illustrating the beneficial motor, non-motor and molecular effects of downregulating CPT1 activity by pharmacological and genetic mechanisms in the SOD1 G93A *in vivo* model mimicking ALS. Further, it present harmful effects of stimulating CPT1 mediated lipid metabolism through high fat diet and corticosterone. Finally, the manuscript presents that downregulation and upregulation of CPT1 activity results in alternations in the gut fecal microbiome in the SOD1 G93A mouse model. This points towards a multisystem effect of downregulation or upregulation of CPT1 activity.

Manuscript II presents data illustrating the beneficial motor, non-motor behaviour and molecular effects of downregulating CPT1 activity by pharmacological and genetic mechanisms (*Cpt1a* P479L mutation) in the rotenone model mouse model mimicking some aspects of PD. Additionally, that pharmacologically downregulation of CPT1 activity is effective in restoring impaired behaviour and genetic expression in the midbrain of *Park2* mice (monogenic autosomal resseciv form of PD) mimicking some aspects of PD. In addition, the manuscript presents that rotenone results in dysbiosis in the fecal gut microbiota and that downregulation of CPT1 activity counteracts some of these pathological alternations. These findings points towards a multisystem effect of downregulating CPT1 activity in the rotenone mouse model mimicking some behaviour and biochemical aspects of PD.

Manuscript III presents data illustrating that C*pt1a* P479L mutated mice are resistant to induction with active immunized EAE compared to normal C57Bl/6J wt mice. In addition the manuscript illustrates that high fat diet exacerbate disease in wt but not C*pt1a* mutated mice. This is underpinned by the decreased demyelination in C*pt1a* mice compared to wt and the lower gene expression of oxidative stress markers. These findings point towards a central role of CPT1A lipid metabolism in this EAE mouse model and possibly the regulation of inflammatory processes.

Manuscript IV presents data illustrating that pharmacological inhibition of CPT1 results in changes in autoantibody-brain-antigen recognition in an EAE rat model, which is consistent with diminished disease activity compared to placebo and the first-line treatment interferon-β. This indicates that downregulation of CPT1 activity affects the production of autoantibodies in the rat EAE model.

Manuscript V summarize findings from the SOD1 G93A, rotenone and EAE models and provides data illustrating that *Cpt1a* P479L mice have changes in their fecal gut microbiota compared to wt. This underpins the systemic role of CPT1A activity. Based on these data, the manuscript presents a systemic platform for how the pathogenic mechanisms in neurodegenerative diseases such as ALS, PD and MS all

are linked to a disruption of metabolic homeostasis. Thereby, it provides a platform for future hypothesis-driven experiments.

In conclusion, this indicates that neurodegenerative diseases have to be recognized from a systemic, multidimensional perspective where dysregulated metabolism plays a pivotal role in the aetiology and progression of neurodegenerative diseases like ALS, PD and MS. In addition, the results indicate that modulation of the dysregulated metabolism, possibly through targeting CPT1, could be a key target in the treatment of neurodegenerative diseases. However, further studies are needed to evaluate more mechanisms and to evaluate the translational perspectives.

CARNITINE PALMITOYL TRANSFERASE 1 – A POTENTIAL TARGET TO RESTORE DYSREGULATED METABOLISM IN NEURODEGENERATIVE DISEASES?

DANSK RESUME

Neurologiske sygdomme er gennem de sidste hundrede år blevet inddelt i sygdomsenheder og klassificeret som eksempelvis motor neuron sygdom, neurodegenerative eller demyeliniserende på baggrund af deres patologiske karakteristika. Men gennem de sidste årtier er det blevet klart, at mange sygdomme, som afficerer central nervesystemet (CNS) har flere fælles patogenese mekanismer samt, at mange CNS sygdomme også afficerer organsystemer udenfor CNS. De neurodegenerative sygdomme amyotrofisk lateral sklerose (ALS), Parkinson's sygdom (PD) samt multiple sklerose (MS) er alle karakteriseret ved eksempelvis inflammation, oxidativt stress, mitokondriel dysfunktion, dysregulering af hypothalamus-hypofyse-binyrerbark-aksen samt ændringer i tarmen og sammensætningen af tarmfloraen. ALS, PD og MS er ydereligere karakteriseret ved dysregulering af metabolismen, specifikt, at glukose metabolismen er nedreguleret mens det indikeres, at lipid metabolismen er opreguleret i såvel CNS samt i periferien.

CNS forbrænder under normale omstændigheder primært glukose, men under patologiske forhold kan CNS skifte til at forbrænde lipider. Lipid metabolismen kan forårsage igangsætning eller forværre sygdomsmekanismer såsom inflammation, oxidativt stress og mitokondriel dysfunktion. Carnitine palmitoyl transferase 1 (CPT1) er lokaliseret til den ydre mitokondrielle membran og er et af nøgle proteinerne i reguleringen af lipid metabolismen, da det faciliterer det første trin i transporten af lange fedtsyrer ind i mitokondriets matrix. I mitokondriets matrix undergår fedtsyrerne β-oxidation, hvorved der dannes acetyl-CoA, som efterfølgende kan bruges i citronsyrecyklus og elektrontransportkæden til at danne ATP. Acetyl-CoA, som bliver dannet via β-oxidation resulterer i negativ feedback til glukose metabolismen via nedregulering af pyruvat dehydrogenase komplekset. Dette resulterer i en ond spiral, som forårsager yderligere opregulering af lipid metabolismen og dermed forværring i patogenesen.
Der findes en befolkningsgruppe, Inuit, som har en høj forekomst af mutationer i *CPT1A* genet, herunder især proline for leucine ved kodon 479 (P479L) mutation, hvilket resulterer i, at CPT1A aktiviteten bliver nedsat til 22 % sammenlignet med ingen mutation. Inuit befolkningen har markant lavere forekomst af en række sygdomme, herunder MS, depression og muligvis også ALS. Dette kunne indikere, at CPT1 spiller en rolle i disse sygdomme.

Baseret på ovenstående har vi testet effekten af at nedregulere samt opregulere CPT1 via farmakologiske, genetiske samt miljømæssige metoder i *in vivo* modeller for ALS (SOD1 G93A model), PD (rotenone samt *Park2* mutation) og MS (eksperimentel autoimmune encefalitis, EAE). Hypotesen har været, at nedregulering af CPT1 aktiviteten leder til langsommere sygdomsprogression eller resistens i forhold til sygdomsinduktion samt nedregulerer sygdomsmekanismer såsom inflammation og oxidativt stress. Derimod leder opregulering til forværret sygdoms fænotype, såvel

adfærdsmæssigt som molekylært. Dette er således blevet undersøgt i følgende 5 manuskripter:

Manuskript I viser, at farmakologisk nedregulering af CPT1 aktiviteten samt genetisk nedregulering via *Cpt1a* P479L mutation i SOD1 G93A modellen leder til langsommere sygdomsprogression baseret på adfærdstests samt dæmper sygdomsmekanismer såsom inflammation, oxidativt stress og leder til potentiel forbedring af den mitokondrielle metabolisme. Herudover illustrerer manuskriptet, at en kost med højt indhold af mættet fedt eller oral administration af corticosterone leder til opregulering af CPT1 medieret lipid metabolisme og øget sygdomsprogression baseret på adfærd samt molekylærer analyser. Endelig viser manuskriptet, at tarmfloraen spiller en rolle i sygdomsinitieringen samt progression i SOD1 G93A modellen og, at denne bliver moduleret ved henholdsvis ned- eller opregulering af CPT1 aktiviteten. Disse fund indikerer, at CPT1 aktiviteten spiller en rolle i multiple organer systemer i forhold til aktivering eller nedregulering af sygdomsmekanismer.

Manuskript II viser, at farmakologisk eller genetisk nedregulering af CPT1 aktiviteten i en toksisk rotenone model for PD leder til henholdsvis reversering af adfærdsmæssige motoriske og non-motoriske symptomer eller resistens mod sygdomsinduktion. Herudover viser manuskriptet, at farmakologisk nedregulering også har positive adfærdsmæssige effekter i en genetisk *Park2* model, som modulerer visse sygdomsaspekter af autosomal resseciv PD. Yderligere viser manuskriptet, at tarmfloraen spiller en rolle i induktionen af den toksiske rotenone model samt, at farmakologisk nedregulering af CPT1 ændrer tarmfloraens sammensætning.

Manuskript III illustrerer, at *Cpt1a* P479L muterede mus er resistente overfor miljømæssigt induceret EAE sammenlignet med vildtype mus samt, at en kost med højt indhold af mættet fedt leder til forværret sygdomsprogression i vildtype mus, men ikke i *Cpt1a* P479L muterede mus. Endelig viser manuskriptet, at den genetiske nedregulering af CPT1A aktiviteten både leder til nedsat demyelinering og oxidativt stress.

Manuskript IV viser, at induktion af EAE i rotter leder til produktion af autoantistoffer i serum imod hjerne autoantigener og, at autoantistof-antigen responset bliver moduleret af farmakologisk nedregulering af CPT1 aktivteten samt behandling med interferon-β. Dette tyder på, at farmakologisk nedregulering af CPT1 leder til potentiel ændring af autoantistof produktion fra B-celler.

Manuskript V opsummerer adfærdsmæssige og molekylærer fund fra manuskript I, II og III samt tilføjer nye fund i både EAE og rotenone model samt viser, at *Cpt1a* ekspression potentielt er epigenetisk reguleret i blod-hjerne-barrier celler. Endelig illustrerer manuskriptet, at *Cpt1a* P479L muterede mus har ændringer i deres tarmflora sammenlignet med vildtype mus, hvilket indikerer, at CPT1A lipid metabolismen direkte og indirekte modulerer tarmfloraens sammensætning. Alle

fundene leder til præsentationen af en systemisk model for hvorledes metabolismen spiller en central rolle i initiering og progression af sygdomsmekanismer, som er impliceret i multiple neurodegenerative sygdomme, inklusiv ALS, PD og MS.

Udfra ovenstående konkluderes det, at neurodegenerative sygdomme bør blive betragtet fra et multisystemisk perspektiv, hvor dysregulering af metabolismen spiller en potentiel, central rolle i ætiologien og progressionen af sygdommene. Yderligere konkluderes det, at CPT1 er et potentielt "target" til at modulere og behandle neurodegenerative sygdomme. Dette kræver dog flere studier, især taget de translationelle udfordringer fra dyremodeller til mennesker i betragtning.

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Michael S. Trabjerg

Michael Sloth Trabjerg, December 2020
Aalborg, Denmark

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LIST OF MANUSCRIPTS

The PhD thesis is based on the following manuscripts:

Manuscript I: Downregulating carnitine palmitoyl transferase 1 affects disease progression in the SOD1 G93A mouse model of ALS. **Trabjerg MS**, Andersen DC, Huntjens P, Oklinski KE, Bolther L, Hald JL, Baisgaard AE, Mørk K, Warming N, Kullab UB, Kroese LJ, Pritchard CEJ, Huijbers IJ, Nieland JDV. [*Submitted*]

Manuscript II: Downregulation of carnitine palmitoyl transferase 1 is highly efficacious in mouse models mimicking Parkinson's disease. **Trabjerg MS**, Andersen DC, Huntjens P, Mørk K, Warming N, Kullab UB, Skjønnemand M-L N, Oklinski MK, Oklinski KE, Bolther, Kroese LJ, Pritchard CEJ, Huijbers IJ, Corthals A, Nieland JDV. [*Submitted*]

Manuscript III: CPT1A plays a key role in the development and treatment of multiple sclerosis and experimental autoimmune encephalomyelitis. Mørkholt AS, **Trabjerg MS,** Oklinski MKE, Bolther L, Kroese LJ, Pritchard CEJ, Huijbers IJ, Nieland JDV. *Sci Rep* **9,** 13299 (2019). https://doi.org/10.1038/s41598-019-49868-6

Manuscript IV: Identification of brain antigens recognized by autoantibodies in experimental autoimmune encephalomyelitis-induced animals treated with etomoxir or interferon-β.Mørkholt AS*, Kastaniegaard K*, **Trabjerg MS**, Gopalasingam G, Niganze W, Larsen A, Stensballe A, Nielsen S, Nieland JD. *Sci Rep* **8,** 7092 (2018). https://doi.org/10.1038/s41598-018-25391-y

Manuscript V: Dysregulation of metabolic pathways by carnitine palmitoyl-transferase 1 plays a key role in central nervous system disorders: experimental evidence based on animal models. **Trabjerg MS**, Mørkholt AS, Lichota J, Oklinski MKE, Andersen DC, Jønsson K, Mørk K, Skjønnemand M-L N, Kroese LJ, Pritchard CEJ, Huijbers IJ, Gazerani P, Corthals A, Nieland JDV. *Sci Rep* **10,** 15583 (2020). https://doi.org/10.1038/s41598-020-72638-8

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CARNITINE PALMITOYL TRANSFERASE 1 – A POTENTIAL TARGET TO RESTORE DYSREGULATED METABOLISM IN NEURODEGENERATIVE DISEASES?

LIST OF ABBREVATIONS

ALS: Amyotrophic lateral sclerosis
ATP: Adenosine Triphosphate
BBB: Blood brain barrier
ChAT: Choline O-acetyltransferase
CNS: Central nervous system
CoA: Coenzyme A
COMT: Catechol-O-methyltransferase
CORT: Corticosterone
CPT1: Carnitine palmitoyl transferase 1
CSF: Cerebrospinal fluid
DDC: Dopamine decarboxylase
EAE: Experimental autoimmune encephalomyelitis
FAs: Fatty acids
FALS: Familial amyotrophic lateral sclerosis
FDA: U.S. Food and Drug Administration
18F-FDG-PET: Fluorodeoxyglucose F 18 positron emission tomography
FTD: Frontotemporal dementia
FUS: Fused in sarcoma
G93A: Point mutation in amino acid position 93 resulting in a switch from glycine to alanin in the *SOD1* gene
GLUT: Facilitative glucose transporter
G6P: Glucose-6-phosphate
HDL: High-density lipoprotein
HPA: Hypothalamic-pituitary-adrenal axis
LDL: Low-density lipoprotein
L-DOPA: Levodopa
MAO-B: Monoamine oxidase type B
MNs: Motor neurons
MS: Multiple sclerosis
NADPH: Nicotinamide adenine dinucleotide phosphate
P479L: Proline for leucine at codon 479 in the *CPT1A* gene
PD: Parkinson's disease
PPARs: Peroxisome proliferator-activated receptors
PPMS: Primary-progressive multiple sclerosis
PRMS: Primary-relapsing multiple sclerosis
PUFAs: Polyunsaturated fatty acids
ROS: Reactive oxygen species
RRMS: Relapse-remitting multiple sclerosis
rRNA: Ribosomal ribonucleic acid
SALS: Sporadic amyotrophic lateral sclerosis
SN: Substantia nigra

SOD1: Superoxide dismutase 1
SPMS: Secondary-progressive multiple sclerosis
TDP-43: TAR DNA-binding protein 43

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CHAPTER 1. INTRODUCTION

In the last century, neurological disorders have been recognized as individual entities and categorized by their pathological characteristics' such as demyelinating-, neurodegenerative-, or motor neuron disease. However, in the last decades, it has become evident that they share several common pathogenic mechanisms, and that most central nervous system (CNS) diseases have disruption of homeostasis in multiple systems outside the CNS. The work presented in this dissertation focuses on the disruption of metabolism and its role in amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and multiple sclerosis (MS). This thesis is based on multiple studies (*manuscript I–V*), which aims to examine the effects of modulating lipid metabolism by down- or upregulation the activity of the gatekeeper molecule of mitochondrial lipid metabolism, carnitine palmitoyl transferase 1 (CPT1) using animal models. In brief, we hypothesized that:

- Downregulation of CPT1 would result in delayed disease progression and that upregulation would result in exacerbation of disease progression in an *in vivo* model mimicking ALS (*manuscript I*).
- Downregulation of CPT1 would result in amelioration of disease in *in vivo* models mimicking some behavioural and biochemical aspects of PD (*manuscript II*)
- Genetic downregulation of CPT1A activity would result in amelioration of disease in an *in vivo* model mimicking some pathological aspects of MS (*manuscript III*)
- Downregulation of CPT1 would result in alternations in autoantibodies towards CNS antigens in an *in vivo* model mimicking some inflammatory and pathological aspects of MS (*manuscript IV*)
- ALS, PD and MS share common pathogenic features and these diseases should be investigated from a multidimensional perspective where dysregulation of metabolism plays a central role (*manuscript V*)

Therefore, in the following sections, a short review of the glucose and lipid metabolism, will be presented. Followed by a concise review of the aforementioned diseases, with a focus on the role of dysregulated metabolism. Thirdly, the introduction will include a description of how CPT1 and thereby lipid metabolism can be modulated. Finally, the introduction will review some general principles of modelling human neurodegenerative diseases.

1.1. THE METABOLISM IN THE CNS

Metabolism is generally defined as "*the sum of biochemical processes in living organisms that either produce or consume energy*" [1]. The synthesis of simple molecules or polymerization into macromolecules is known as anabolism, whereas the utilization of glucose, fatty acids (FAs), and amino acids to produce energy is known as catabolism [1]. During normal homeostatic balance, the body has a constant matching between the oxidation of glucose and FAs, known as the Randle cycle [2]. In other words, *supply* and *demand* have to be kept in balance.

1.1.1. GLUCOSE METABOLISM

The brain accounts for 2% of the bodyweight but accounts for 20% of the glucose catabolism, making it the main glucose-utilizing organ in the body [3]. Glucose has multiple functions in the CNS such as serving as fuel for the CNS by the generation of Adenosine Triphosphate (ATP), synthesis of neurotransmitters, neurotransmission, and the basis for maintenance of neuronal- and non-neuronal cells [3].

Glucose is transported across the endothelial membrane at the highly selective blood-brain-barrier (BBB) by the facilitative glucose transporter 1 (GLUT1) and into the extracellular fluid compartment [3]. GLUT1 also facilitates the subsequent transport into oligodendrocytes, microglia, and astrocytes, the non-neuronal cells of the CNS. GLUT3, which has a higher transport rate compared to GLUT1, is primarily responsible for the transport into neurons [4]. This ensures a sufficient amount of glucose for the neurons under a variety of conditions. [3]. However, many different GLUTs exist, and several of them have different localizations- and mechanisms within-, and outside the CNS [4,5].

Glucose can undergo metabolism by several pathways but the common first step is the phosphorylation of glucose to glucose-6-phosphate by hexokinase-1 [3]. Glucose-6-phosphate (G6P) can then be used for processes such as ATP production by the glycolytic pathway, nicotinamide adenine dinucleotide phosphate (NADPH) production by the pentose phosphate pathway, or glycogenesis for the storage of energy [3]. Within the glycolytic pathway, G6P is converted into fructose-6-phosphate, followed by conversion into pyruvate. Pyruvate can then enter the mitochondria, be converted into acetyl coenzyme A (CoA), and catabolized in the Krebs cycle [2].

The glucose metabolism is strictly regulated by multiple mechanisms including, but not limited to, hormones (e.g. insulin, glucagon), hypothalamic brain areas (e.g. arcuate, dorsomedial, and paraventricular nucleus, ventromedial hypothalamus), and the gut microbiota [3,6,7]. Other central regulators are the peroxisome proliferator-

activated receptors (PPARs), which are nuclear receptor proteins functioning as transcription factors [8].

1.1.2. LIPID METABOLISM

The CNS contains the second-highest amount of lipids next to adipose tissue and was considered too dependent solely on glucose metabolism for the last century, but it has been gradually revealed that lipids become an alternative source under specific conditions [9–11]. In this regard, it has become evident that the CNS can utilize lipids, especially under low glucose concentrations, or aglycemia [12–15].

Overall, lipids can be divided into five subcategories including FAs, triglycerides, sterol lipids, sphingolipids, and phospholipids [9]. 5% of all the human genes are associated with lipid metabolism, which highlights its importance in biological functions [9]. FAs are essential components in all lipid categories and contain a carbon chain, which terminates in carboxylic acid group [16]. FAs can be divided into subgroups based on the length of the carbon chain [9]. Short-chain FAs have 2–6, medium-chain FAs 7-14, long-chain FAs 15–18, and very-long FAs have 19 or more carbon molecules [9,16]. FAs with different length of the carbon chain have different biological functions, and localization [9]. Short-chain FAs are synthesized in the intestines by the gut microbiota, whereas the long-chain FAs constitute a major part of the diet [16]. FAs are divided into saturated, and unsaturated. The saturated FAs carbon chain is saturated with the hydrogen bonds, whereas unsaturated FAs are defined by the presence of double bonds in the carbon chain. Based on the number of double bonds, unsaturated FAs are subdivided into mono-, or polyunsaturated FAs (PUFAs) [9]. Polyunsaturated FAs constitute an essential part of the cell membranes and regulate multiple processes within the CNS including neurotransmission, inflammation and cell survival [11]. Examples of important PUFAs are arachidonic acid and docosahexaenoic acid. Saturated, and monounsaturated FAs can be synthesized within the CNS, however PUFAs have to be supplied from the periphery [11].

The metabolism of lipids are divided into the exogenous, endogenous, and reverse cholesterol pathway [8]. Lipids that enter the brain are derived from multiple sources in the blood including lipoproteins and unesterified FAs [11]. FAs are taken up by the CNS endothelial cells by multiple mechanisms such as low-density lipoprotein receptors, major facilitator superfamily domain-containing protein 2A, or CD36 [11]. Following the uptake into the brain endothelial cells, FAs are transported across the BBB, and into the brain by passive diffusion or distinct transport proteins such as FAs transport proteins, depending on the molecular size [11,17]. Long-chain and very-long-chain FAs are primarily transported into the brain by FAs transport protein 1 and 4 [17]. These

proteins have acyl-CoA synthase activity, which converts the FAs to fatty acyl-CoA, and thus "trap" them within the cell [11,17].

1.1.3. CARNITINE PALMITOYL TRANSFERASE SYSTEM

Mitochondria are the primary site for lipid metabolism [18]. However, very long-chain FAs have to be broken down by the peroxisomes before they can undergo metabolism in the mitochondria [8,19]. The outer mitochondrial membrane is impermeable to fatty acyl-CoA, and therefore the carnitine palmitoyl transferase (CPT) system has to be used [20]. The first step is the conjugation of carnitine to fatty acyl-CoA forming acyl-carnitine. This process takes place at the outer mitochondrial membrane and is facilitated by the CPT1 enzyme, making the CPT1 a gatekeeper molecule [20]. Acyl-carnitine is then transported across the inner mitochondrial membrane, and into the matrix by the carnitine/acyl-carnitine translocase [20]. In the mitochondrial matrix, CPT2 removes the carnitine from acyl-carnitine and reconverts it to acyl-CoA [20]. The acyl-CoA located in the matrix are metabolized by β-oxidation to form the end product acetyl-CoA, which are subsequently used in the Krebs cycle (**Figure 1**) [18,20,21]. The production of acetyl-CoA from β-oxidation results in downregulation of the conversion of pyruvate into acetyl-CoA, thereby downregulating glucose metabolism [2]. Carnitine is relocated to the cytosol and can be reused in the carnitine shuttle [20]. CPT1 is reversibly inhibited by malonyl-CoA. Moreover, CPT1 is regulated by a variety of mechanisms including PPARs, and insulin [8,18].

CPT1 exists in three different isoforms: CPT1A, CPT1B, and CPT1C. CPT1A (or CPT1L) is expressed in most of the tissues in the body including the liver, adipose tissue, heart, pancreas, and CNS, and *CPT1A* is located at chromosome 11q13.1-q13.5 [22,23]. CPT1B (or CPT1M) is expressed in heart and skeletal tissue, and *CPT1B* is located at chromosome 22q13.3-qt [22,23]. CPT1C is only found in the CNS and is indicated to be an energy sensor, and possibly involved in cognition [22,24,25]. CPT1C is located on the chromosome 19q13.33 [22]. CPT1A and CPT1B are both found at the outer mitochondrial membrane, as gate-keeper molecules for β-oxidation, whereas CPT1C is located at the endoplasmic reticulum [18,24]. The principles of how the activity of the CPT1 system can be modulated will be reviewed in section 1.6 in the introduction.

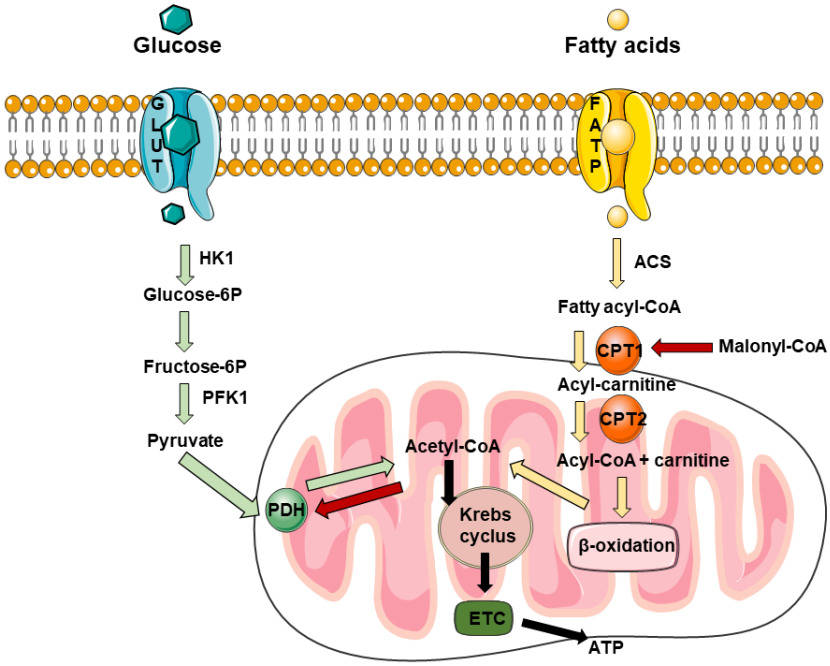

Figure 1: Metabolism of glucose and lipids. Glucose is transported across the cell membrane and into the cell by facilitative glucose transporter (GLUT) and converted into glucose-6-phosphate (Glucose-6P) by hexokinase-1 (HK1) [3]. Glucose-6P is converted into fructose-6-phosphate (Fructose-6P) followed by conversion to pyruvate by phosphofructokinase-1 (PFK1) [3]. Pyruvate is transported into the mitochondrial matrix and converted into acetyl-CoA by the pyruvate dehydrogenase complex (PDH) and subsequently used in the Krebs cyclus and electron transport chain (ETC) to generate ATP [3]. Fatty acids are transported into the cell by fatty acid transport proteins (FATP) and converted into fatty acyl-CoA by acyl-CoA synthase (ACS) [11]. Subsequently, fatty acyl-CoA is transported across the outer mitochondrial membrane by carnitine palmitoyl transferase 1 (CPT1) by conversion into acyl-carnitine, due to conjugation, which then is transported into the inner matrix by carnitine palmitoyl transferase 2 (CPT2) and converted to acyl-CoA + carnitine [20]. Carnitine is then shuttled back to the cytosol and reused. Acyl-CoA is used in the β-oxidation to generate acetyl-CoA followed by processing in the Krebs cyclus and ETC to generate ATP [20]. Acetyl-CoA produced by β-oxidation exerts negative feedback to PDH, and thereby downregulates glucose metabolism [2]. CPT1 is inhibited by negative feedback by malonyl-CoA [20]. Inspired and based on figure 1 in [26]. Illustration elements was obtained from Servier Medical ART with license; https://creativecommons.org/licenses/by/3.0/. No changes were made to the elements.

1.2. DISEASE MECHANISM IN NEURODEGENERATIVE DISEASES

ALS, PD and MS and other neurodegenerative diseases share multiple pathogenic mechanisms including oxidative stress, glutamate excitotoxicity, mitochondrial dysfunction, neuroinflammation, upregulation of the hypothalamic-pituitary-adrenal axis (HPA), disrupted myelin homeostasis and alternation in the gut microbiota leading to dysbiosis and leaky gut (**Table 1**). In the following sections (1.3, 1.4 and 1.5) ALS, PD and MS will be reviewed with regard to epidemiology, clinical symptoms, aetiology, pathogenesis and the U.S. Food and Drug Administration (FDA) approved treatments to highlight common features of the pathogenic mechanisms.

Table 1: Description of common pathogenic mechanisms implicated in neurodegenerative diseases.

Mechanism	**Definition**
Oxidative stress	Oxidative stress is defined as an imbalance between the production, and accumulation of toxic, reactive molecules such as superoxide, hydrogen peroxide, 4-hydroxy-2-nonenal (4-HNE), reactive oxygen species (ROS), and reactive nitrogen species (RNS) and the removal of these molecules by antioxidants such as Cn-Zn superoxide dismutase 1 (SOD1), heme oxygenase 1 (HO1), vitamin E and homocysteine [27,28].
Glutamate excitotoxicity	Excitotoxicity is defined as a pathological high increase in otherwise necessary, and safe neurotransmitters [29]. Glutamate is the primary excitatory neurotransmitter in the CNS and binds to postsynaptic neurons [29]. Under homeostatic conditions glutamate is removed from the synaptic cleft by astrocytes, ending the signal [29]. If this pathway is disrupted, glutamate leads to overstimulation of several receptors, and thereby toxic intracellular transport of calcium [29]. This results in the activation of enzymes such as proteases, and phospholipases, which cause damage to intracellular organelles [30]. Moreover, the high calcium influx can lead to oxidative stress and mitochondrial dysfunction [29].

Mitochondrial dysfunction	Mitochondrial dysfunction is defined as loss of function of the electron transport chain and thereby impaired production of energy molecules, including ATP, which can be initiated by mechanisms such as a decrease in numbers of mitochondria, and impairment in the availability of substrates [31]. Normal mitochondrial respiration results in ROS-production as a byproduct, which requires detoxification by antioxidants [28]. Additionally, mitochondrial DNA lacks protective histones, which makes them extra vulnerable for mutations and thereby defective biogenesis [28]..
Neuroinflammation	Neuroinflammation is defined as the response from microglia, astrocytes, and peripheral immune cells (monocytes, lymphocytes, and neutrophils) that enter, and interact with cells within the CNS during pathological circumstances such as neurodegeneration, injury, or infection [32–34].
HPA-axis disruption	Any imbalances to an organism's homeostasis elicit a complex stress response that causes activation of the neuroendocrine and autonomic system [35]. One of the essential systems in the stress response is the HPA axis [35]. During acute stress, such as critical sickness, the stress response is beneficial for survival. However, prolonged stress, due to psychological or physiological reasons, causes an over activation of the HPA-axis resulting in high levels of glucocorticoids. [35] High levels of glucocorticoids in turn result in insulin resistance, which forces metabolism towards lipolysis [36]. Moreover, prolonged high levels of cortisol eventually result in glucocorticoid receptor resistance, which as a result fails to downregulate the inflammatory response [37,38]. In addition, stress can induce the production of prostaglandin E2, which activates and attracts the innate and adaptive immune system [39].
Myelin homeostasis	Myelin is a multilayer wrapping that insulates the nerve axons, which is essential for fast conduction of nerve impulses, and metabolic support for neurons [40]. Myelin is synthesized by oligodendrocytes in the CNS, and by Schwann cells in the peripheral nervous system [40]. Myelin is

	composed of proteins and lipids. The major proteins in myelin include myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP). Lipids are essential in the generation, and function of myelin. The major lipids in myelin include high amounts of long-chain FAs, glycosphingolipids, and cholesterol. [41] Myelin homeostasis can be disrupted by multiple mechanisms such as mutations, and autoimmune reactivity against myelin proteins causing demyelination. [41]
Gut microbiota	In the last decade, multiple studies have indicated a connection between the gut, and the brain, known as the gut-brain-axis. Moreover, the microbiota in the gut is indicated to be dysregulated in several neurodegenerative diseases, including ALS, PD, and MS [42,43]. Short-chain FAs, such as butyrate, are produced by the gut microbiota during fermentation of fibres [44]. These are important for the maintenance of intestinal homeostasis, and modulation of the immune system [45].

1.3. AMYOTROPHIC LATERAL SCLEROSIS

ALS is a fatal, progressive neurodegenerative disease characterized by clinical, and genetic heterogeneity, which affects both motor and extra-motor systems [46]. The disease typically results in death due to respiratory failure within 3 – 5 years from diagnosis [47]. It is pathologically characterized by the death of upper motor neurons (MNs) in the brain, and lower MNs in the brainstem, and medulla spinalis [47]. This results in the scarring of the lateral tracts in the medulla spinalis, which is accompanied by the accumulation of deposits of aggregated proteins in the MNs [48]. The death of upper MNs result in muscle stiffness, and spasticity, whereas the death of lower MNs leads to muscle atrophy [47]. ALS was originally viewed as a neuromuscular disease, but approximately 50 % of the patients have behavioural abnormalities and cognitive impairment including frontotemporal dementia (FTD) [46]. Typically, the disease presents with focal symptoms but then spreads to other parts of the nervous system. ALS can affect every voluntary muscle, except for extraocular and sphincter muscles. Thereby, making the clinical presentation heterogeneous, and diagnosis difficult [46,49,50]. ALS can overall be divided into the following clinical subtypes: Classic ALS (70%), ALS-FTD (5-15%), isolated bulbar involvement (5%), restricted phenotypes of ALS (10%), and rare phenotypes (3%) [46].

The median incidence of ALS is 1 – 2 cases/year per 100,000 in Europe and the United States, with a median prevalence of 3 – 5 cases per 100,000 [47,51]. The mean age of disease onset is 55 years old, and the risk of ALS increases until the age of 75 [51]. ALS exists in a familial (fALS), and a sporadic form (sALS) (defined as no family history of ALS) [48]. FALS accounts for approximately 10% of all cases, whereas sALS accounts for 90 % of all cases [47]. SALS has a male/female ratio of 2:1, whereas the ratio for fALS approaches 1:1 [47].

Mutations in the gene coding for superoxide dismutase 1 (*SOD1*) was the first gene linked to ALS in 1993, which resulted in the generation of the model with a point mutation in amino acid position 93, inducing a switch from glycine to alanin (G93A) in the *SOD1* gene, known as SOD1 G93A transgenic mice [52,53]. In the last two decades, more than 30 genes have been linked to ALS, including *TARDBP*, *C9ORF72*, and *FUS*, most with a dominant penetrance [48]. The mutations can generally be divided into three groups 1) genes that are implicated in proteostasis, and quality control 2) genes affecting RNA stability, metabolism, and function, and 3) genes that regulate cytoskeletal dynamics in MNs [48]. These mechanisms are reviewed in depth in [48]. Even though fALS accounts for a small proportion of all cases, several of the genes linked to fALS, have also been found in patients with sporadic disease [54]. The genetic mutations result in the deposition of different inclusions in the MNs. The most prominent inclusion is the TAR DNA-binding protein 43 (TDP-43), which are found

both in fALS and sALS [47]. However, *SOD1* and fused in sarcoma (*FUS*) mutations result in deposition of SOD1, and FUS aggregates, respectively [48].

Despite intensive research and the development of innovative methods, the aetiology of ALS remains elusive. The attempts to establish common risk factors have overall been inconclusive [47]. Nonetheless, risk factors such as military service, smoking, exposure to heavy metals, pesticide exposure, human endogenous retrovirus K, trauma, and repetitive concussion are associated with an increased risk of ALS [47]. However, ALS could develop based on a multistep process, requiring certain gene-environment interactions [55]. Despite the unknown aetiology, multiple pathogenic mechanisms were discovered during the last decades. It's now evident that the disease arises not only due to mutations in MNs, but also glial cells (microglia, oligodendrocytes, and astrocytes) [48]. Additionally, it has been hypothesized that ALS could arise from the muscles, and spread by retrograde mechanisms to the MNs [56]. Oxidative stress, glutamate excitotoxicity, mitochondrial dysfunction, neuroinflammation, dysregulation of the HPA axis, demyelination, and disruption of the gut microbiota are all linked to the development and/or progression of ALS (**Table 2**).

Table 2: Overview of pathogenic mechanisms implicated in ALS, with examples.

Mechanism	**Findings in ALS**
Oxidative stress	Increased oxidative damage to proteins in the spinal cord of ALS patients [57]. Increased oxidative damage to proteins, and lipids in the brain in both sALS, and fALS [58]. Decreased NADPH-oxidase 2 (NOX2) activity is correlated with prolonged survival [59]. Lipid peroxidation is significantly increased in SOD1 G93A mice from day 30 and onwards [60]. Prostaglandin E2 can induce the formation of ROS and are increased in ALS patients [61]. 4-hydroxy-2-nonenal (4-HNE) is significantly increased in serum, and spinal cord tissue from ALS patients [62], and SOD1 G93A mice [63]. 4-HNE induces the deposition of TDP-43 inclusions *in vitro* [64]. Antioxidants such as ascorbic acid, uric acid, and homocysteine are elevated in cerebrospinal fluid (CSF) from ALS patients [65]. Microglia with mutated SOD1 activates NOX2, and thereby the production of extracellular superoxide [48].

Glutamate excitotoxicity	Plasma and CSF glutamate levels are significantly higher in ALS patients compared to healthy controls [66,67]. SOD1 G93A mice have increased levels of glutamate in the CNS [68]. SOD1 G93A mice have impaired reuptake due to downregulation of the glutamate transporter, GLT-1, in astrocytes, by the SOD1 mutation [69]. In accordance, CSF from ALS patients is highly toxic to motor neurons *in vitro* through AMPA, and kainite receptors [70]. The human form of GLT-1, excitatory amino acid/glutamate transporter 2 (EAAT2), is impaired in cell models and ALS patients [29]. The downregulation of EAAT2 could be mediated by multiple factors including the pro-inflammatory cytokine TNF-α [71] and deprivation of glucose under low oxygen [72].
Mitochondrial dysfunction	CSF from sALS patients induce mitochondrial dysfunction *in vivo* by downregulation of mitochondrial proteins associated with energy production and stimulates apoptosis [73]. SOD1 G93A MNs have dysfunctional mitochondrial fusion, axonal transport, smaller size, decreased density, impaired membrane potential and mislocalization [74]. MN-like cell line transfected with SOD1 G93A mutation is characterized by impaired respiration and membrane potential [75]. FALS and SOD1 G93A mutated mice have dysfunctional mitochondrial complex 1 activity [76]. SOD1 G93A mice have impaired mitochondrial biogenesis [77]. The SOD1 mutation results in a metabolic shift towards lipid metabolism [78–80]. The metabolism of lipids requires more oxygen compared to glucose metabolism, which results in production of reactive oxygen species leading to oxidative stress and exacerbation of mitochondrial dysfunction [15].
Neuroinflammation	In ALS, microglia, astrocytes, and innate immune cells play a major role in the neuroinflammatory response [32]. However, also T-cells, including CD4, CD8 and regulatory T-cells, play a role [81]. SOD1 G93A silencing in microglia has positive effects *in vivo* and *vitro* [48]. Increased levels of the pro-inflammatory cytokine IL-6 induces disruption of BBB [82]. ALS patients have in the blood increased levels of multiple inflammatory cytokines, including TNF-α, IL-6, IL-1β, and IL-8 compared to healthy controls [83]. ALS

	patients have significantly increased numbers of cytotoxic CD8 positive T-cells, natural killer T-cells and significantly reduced levels of regulatory T-cells [81]. Self-reactive CD8 positive T-cells infiltrate the CNS and cause the death of MNs in SOD1 G93A transgenic mice, which is ameliorated when CD8 positive T-cells are ablated [84]. Both sALS and fALS patients have significantly elevated levels of IL-17A in serum compared to healthy controls [85]. Further, sALS patients have increased levels of IL-17A positive CD8 and mast cells together with TNF-α and IL-1β positive macrophages in the spinal cord [85]. SOD1 G93A mice have increased levels of GFAP and IBA1 positive cells in the spinal cord and increased inflammatory gene expression compared to non-transgenic mice [86] (*manuscript I*).
HPA-axis disruption	ALS patients have significantly increased levels of morning cortisol compared to healthy controls, which correlate with disease progression [87]. ALS patients have disrupted cortisol awakening response, which correlates with a poor clinical status [88]. ALS patients have a loss of circadian rhythm of cortisol levels [89]. SOD1 G93A mice exposed to chronic restraint stress have significantly increased corticosterone, which is associated with a more aggressive disease progression, a higher level of inflammatory cytokines, and decreased survival [90]. The HPA-axis is dysregulated in the Wobbler mouse model, mimicking ALS [91]. Glucocorticoids result in a more severe MN pathology in the TDP-43 mouse model mimicking ALS [92]. Additionally, administration of corticosterone by oral gavage results in a more severe disease progression, possibly due to metabolic disruption and inflammation (*manuscript I*).
Myelin homeostasis	ALS patients have decreased myelin staining in the anterolateral columns in the medulla spinals with macrophage infiltration [93]. Ablation of mutant SOD1 from oligodendrocytes results in delayed disease onset and increased survival in SOD1 G93A mice [48]. Spinal cord myelin samples from SOD1 G93A transgenic rats have a decrease in the phospholipid level, cholesterol, and cerebrosides [94]. Oligodendrocytes in the ventral grey matter

	in SOD1 G93A transgenic mice have morphological changes including swelling of the cell body and a reactive morphology [95]. The number of these pathological oligodendrocytes increases as the disease progresses and apoptotic oligodendrocytes were compensated by oligodendrocyte precursor cells, which expressed less myelin basic protein [95]. Zebrafish selectively expressing SOD1 mutant oligodendrocytes were characterized by anxiety-like behaviour, learning impairment, and motor dysfunction [96]. Moreover, mutant SOD1 disrupted the myelin sheets, and induced MN death [96]. The disruption of myelin could be due to increased turnover of lipids due to a metabolic shift, inflammation and oxidative stress (*manuscript I, manuscript III*).
Gut microbiota	SOD1 G93A transgenic mice have disruption of tight junctions in the gut, increased permeability and IL-17A levels in the gut [97]. SOD1 G93A mice have a shift in the microbiome compared to healthy control mice [97,98] (*manuscript I*). ALS patients have decreased microbiome diversity, increased inflammatory stool markers, low levels of short-chain FAs and a shift in their gut microbiota [99]. SOD1 G93A mice have changes in their gut microbiome before clinical disease onset, muscle atrophy, and inflammation in the spinal cord [98]. Butyrate supplementation to SOD1 G93A mice restore gut integrity and increases survival [100]. Further, butyrate diminishes aggregation of SOD1 proteins [100].

Interestingly, several studies indicate that ALS is associated with hypermetabolism, which is characterized by dysregulation of metabolism in the CNS, and periphery [101]. ALS patients have significantly higher levels of pyruvate in the cerebrospinal fluid (CSF) compared to healthy controls [102]. Dysregulated glucose metabolism is associated with disease progression [103,104]. ALS patients have indications of increased lipid metabolism in the CNS [105]. Pathologic increased low-density lipoprotein / high-density lipoprotein (LDL/HDL) ratio in the serum is associated with an increased incidence of ALS [106]. *In vivo* models mimicking fALS indicate that the disease is characterized by increased clearance of lipids in the periphery, and insulin resistance [107,108]. SOD1 mice show upregulated lipid metabolism and decreased glucose

metabolism in the CNS and muscles before disease onset, which is associated with the upregulation of CPT1A and CPT1B [78–80,109]. The upregulation of glucose metabolism in muscles following exercise in SOD1 mice has shown protective effects [80]. In accordance, lipid metabolism is upregulated in the spinal cord of SOD1 mice [79]. Moreover, *C9orf72* mutations are associated with increased lipid metabolism and oxidative stress [110,111]. Serum lipids are changed in ALS patients compared to healthy controls [112]. These, and other findings, indicate that a shift from glucose to lipid metabolism could play a central role in the development, and progression of ALS.

Despite intensive research since 1993, no cure exists, and only two drugs are approved by the FDA for the treatment of ALS at the moment (**Table 3**). Riluzole was approved in 1995 as the first drug to treat ALS. However, the drug was not tested in preclinical models and only extends survival by a few months in randomized clinical trial studies [113,114]. In 2017, more than 20 years after the approval of riluzole, edavarone was approved by the FDA to treat ALS [115]. Edavarone only shows effect in a subset of ALS patients with early-stage disease, and approval of edavarone in Europe has been retracted [116]. Thus, there is a demand for novel therapeutics to treat ALS.

Table 3: FDA approved drugs for the treatment of ALS.

Drug	**Target**	**Mode of action**
Riluzole	Glutamate	The mode of action of riluzole is not completely understood. It is indicated to block the presynaptic release of glutamate [114]. However, other antiglutamatergic drugs have failed to show effect in ALS. Further, it is indicated to upregulate glucose metabolism *in vitro* [113].
Edavarone	Oxidative stress	Edavarone reduces toxic levels of superoxide- and hydroxyl radicals and diminishes the peroxidation of lipids by electron transfer [117].

1.4. PARKINSON'S DISEASE

PD is a progressive neurodegenerative disease characterized by the death of dopaminergic neurons in the substantia nigra (SN), and non-dopaminergic neurons [118]. It is distinguished by clinical heterogeneity and results in severe morbidity due to motor- and non-motor symptoms [119]. Non-motor symptoms such as olfactory impairment, cognitive dysfunction, depression, disrupted sleep patterns, obstipation, fatigue, and pain often precede motor symptoms by more than a decade [120]. Classical motor symptoms include bradykinesia, resting tremor, rigidity, and impairment of gait [119]. PD is associated with increased mortality compared to the general population as the disease progresses [121].

The prevalence of PD varies considerably with regards to geography e.g. prevalence of 66-1500 cases per 100.000 in Europe compared to 10-43 cases per 100.000 in Africa [119]. The incidence of PD is estimated to be 10-18 per 100.000 per year [122]. The mean onset of the disease is at an age of 70 [123]. PD can overall be categorized as tremor-, or non-tremor dominant, and exists in an idiopathic and a familiar form [123]. 85 – 90 % of all PD cases are idiopathic [123]. The diagnosis is based on clinical examination, and the diagnosis of idiopathic PD was formalized by "*UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria"* [124], resulting in 90% diagnostic accuracy [124,125]. Most patients receive a diagnosis of possible PD because post mortem findings of depigmentation of SN, loss of neurons, and the presence of Lewy body inclusions are required for a final diagnosis [119]. Lewy bodies are cytoplasmic depositions primarily composed of α-synuclein (α-syn) aggregates [118]. The normal physiologic function of α-syn is not completely understood, but it is indicated to play a role in mitochondrial function, vesicle dynamics, and intracellular trafficking [118]. α-syn is initially unfolded but has the capacity to misfold, and form α-syn oligomers and protofibrils, which lead to a toxic cascade causing death of dopaminergic neurons [126]. The misfolding and accumulation of α-syn can be caused by several processes including overproduction, disrupted degradation, and mutations, which increases the risk of misfolding [118]. Initially, α-syn is localized in the brainstem, olfactory system, and possibly also the gut and propagate as the disease progresses and spreads to areas such as the limbic, and neocortical brain regions [118,127,128].

Six genes have been confirmed as the cause of inherited monogenic PD: *SNCA*, *LRRK2*, *PARK2*, *PINK1*, *DJ1*, and *UCHL1* [123]. Mutations in *PARK1* (*SNCA*) was the first gene linked to PD [123]. Autosomal dominant mutations in *PARK8* (*LRRK2*) are the most frequent cause of late-onset PD, and mutations are highly prevalent in idiopathic PD [129]. Whereas autosomal recessive mutations in *PARK2* (*Parkin*) are the most prevalent mutated gene in early-onset PD and idiopathic PD with onset below the age of 50 years [123]. PD patients with *Parkin* mutations typically have an onset of disease

between 35 – 45 years and are in most cases not associated with the presence of Lewy bodies [129,130].

The aetiology of PD is not understood but multiple risk factors have been identified. Age is the largest risk factor for PD, and the incidence increases exponentially until the age of eighty [119]. Males have the highest risk of developing PD with a 3:2 male to female ratio [119]. Ethnicity is also associated with the risk of PD as whites have increased risk compared to Asians, and Blacks [131]. Additionally, environmental risk factors such as pesticide exposure and head injury are associated with increased risk of developing PD [119,132–134]. Further, various genetic risk factors, including mutations in *LRRK2*, are confirmed [119,129]. Multiple mechanisms are associated with the pathogenesis of the development, and progression of PD, including α-syn aggregation, oxidative stress, mitochondrial dysfunction, neuroinflammation, disruption of the HPA-axis, and alternations in the gut microbiota (**Table 4**).

Table 4: Overview of pathogenic mechanisms implicated in PD, with examples.

Mechanism	**Findings in PD**
α-synuclein	α-synuclein (α-syn) is present in PD patients and spread to different brain regions as the disease progresses in a prion-like manner [127]. *SNCA* A53T mutation in mice results in severe motor deficits, and the formation of Lewy bodies [135]. α-syn fibrils inoculated in the gut results in gastrointestinal defects, and exacerbation of pathology in the midbrain, including motor defects, in aged mice [128]. PD patients have significantly elevated levels of autoantibodies against α-syn compared to healthy controls, and Alzheimer's disease patients [136]. The level of α-syn increases during aging, and is associated with the death of dopaminergic-neurons in SN [137]. Aging is associated with decreased function of the lysosomal autophagy system, which is responsible for the degradation of α-syn [138].
Oxidative stress	The SN has decreased levels of glutathione resulting in decreased antioxidant defense [139]. Mutations in *DJ1*, which codes for an antioxidant, results in early-onset PD due to oxidative stress [140,141]. The SN contains the highest amount of iron [142]. Under normal circumstances, iron is equally present in a reduced (Fe^{2+}), and an oxidized form (Fe^{3+}) [142]. However, PD patients have an increased amount of reduced

	iron, which can induce toxicity [142]. Induced pluripotent stem cell-derived human midbrain neurons with *Parkin* mutations have increased levels of oxidative stress due to dopamine oxidation [143]. Pesticide toxins induce PD-mimicking disease *in vivo* by oxidative stress, and mitochondrial dysfunction mechanisms [135] (*manuscript II, V*).
Mitochondrial dysfunction	Mitochondria are dysfunctional in a variety of neurodegenerative diseases including PD [144]. Inhibition of complex 1 in the electron transport chain induces PD-like disease *in vivo* [145]. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) is a master regulator in mitochondrial biogenesis and is decreased in the SN of PD patients [146]. Additionally, the upregulation of PGC1α results in lower levels of α-syn in *vitro* [147]. Mitochondrial quality control is disrupted by PD-related genetic mutations [148]. Neurons derived from *PARK2* patients are characterized by mitochondrial dysfunction, and oxidative stress [149].
Neuroinflammation	Neuroinflammation is indicated to play a central role in several neurodegenerative diseases, including PD [34]. Post mortem analyses of PD brains show increased levels of reactive microglia in the SN [150]. Microglia show increased expression of IL-6 and TNF-α [151]. Inflammatory interleukins, including IL-6, are increased in CSF from PD patients [152,153]. Multiple inflammatory markers are increased in serum, supernatants from *in vitro* studies, and leukocytes from PD patients (reviewed in [154]).
HPA-axis disruption	Multiple studies have found elevated levels of cortisol in PD patients compared to healthy controls [155]. Additionally, high cortisol levels are associated with worse motor performance [155]. Glucocorticoid receptors are indicated to be deregulated in PD, which could result in inflammation and death of dopaminergic neurons [156,157].
Gut microbiota	PD patients have significant alternations in their gut microbiota compared to healthy controls [43,158–160]. Lower Lachnospiraceae and higher Lactobacillaceae and Christensenellaceae are associated with aggravated disease progression based on the motor, and non-motor symptoms [161]. Gut microbiota is required for the development of motor

	symptoms, PD-like pathology, and neuroinflammation in α-syn overexpressing mice, which is exacerbated by fecal transplants from PD patients [162]. Chronic blockade of mitochondrial complex 1 induces changes in gut microbiota, PD-like disease symptoms, gastrointestinal dysfunction, and pathology [163].

Additionally, G6P dehydrogenase is decreased in the putamen in sporadic PD patients [164], which is consistent with decreased glucose metabolism based on fluorodeoxyglucose F 18 positron emission tomography (18F-FDG-PET) brain scans in PD patients [165,166]. Interestingly, Eggers et al. (2018) indicate that non-tremor dominant PD have decreased glucose metabolism in the striatum compared to tremor dominant PD patients [167]. The reduced glucose metabolism in the brain is associated with memory impairment in PD patients [168]. Furthermore, diabetes is associated with decreased striatal dopamine levels and high α-syn levels in the CSF [169]. PD patients with diabetes have a faster progression of motor and cognitive symptoms [169]. In accordance with reduced glucose metabolism, PD patients have increased β-oxidation metabolites in the urine [170,171] and serum [172]. Intriguingly, α-syn expression results in changes in lipid profiles, including increased oleic acid, in several *in vitro* models [173]. Diminishing the increased levels of FAs resulted in the amelioration of α-syn oligomerization [173]. The pesticide, rotenone, leads to the induction of PD-like disease *in vitro*, and *in vivo* in conjunction with disrupted glucose metabolism and increased CPT1 activity [174,175] (*chapter 3, manuscript II*). This indicates that a disrupted glucose metabolism could play a pivotal role in the development, and possibly progression of PD.

The death of dopaminergic-neurons in SN leads to decreased levels of dopamine in the striatum, which results in the classic motor features of PD [118]. Thus the pharmacological treatment of PD is based on dopamine substitution in combination with increasing the bioavailability of active dopamine in the CNS. Dopamine cannot cross the BBB and is therefore administered as levodopa (L-DOPA). L-DOPA is metabolized in the periphery by dopamine decarboxylase (DDC), catechol-O-methyltransferase (COMT) and monoamine oxidase type B (MAO-B) in the CNS [118]. At the moment, several drugs are approved by the FDA for the treatment of the motor features in PD (**Table 5**). L-DOPA is the golden standard for the treatment of PD. However, randomized double blinded placebo controlled clinical trials have shown that L-DOPA does not modify the disease, but only relives symptoms [176–178]. Additionally, PD is associated with psychiatric symptoms including hallucinations, which can be exacerbated by dopamine treatment [179]. Thus, there is a demanding need for novel pharmacological targets in PD.

Table 5: FDA approved drugs for the treatment of PD.

Drug	**Target**	**Mode of action**
Levodopa-carbidopa	Dopamine receptors and peripheral dopamine decarboxylase (DDC)	Carbidopa inhibits DDC in the periphery and thereby increases the amount of active dopamine in the CNS [180].
Apomorphine	Dopamine receptor 1-5	Stimulates dopamine receptors in the brain. [181]
Pramipexole	Dopamine D2/D3 receptor	Pramipexole stimulates D2-D3 dopamine receptors reducing motor symptoms in PD. [182]
Ropinirole	Dopamine D2 receptor	Ropinirole is a non-ergoline D2 receptor agonist. [183]
Rotigotine	D3/D2/D1 receptor	Rotigotine is a non-ergoline dopamine antagonist, which stimulates D3/D2/D1 receptors [184]. It is delivered by a transdermal delivery system. [184]
Istradefylline	Adenosine A2A receptor	Istradefylline inhibits Adenosine A2A receptors in the striatum and thereby reduces off-periods in late-stage PD [185]. It is used as add-on therapy in conjunction with levodopa-carbidopa [185].
Entacapone	Catechol-O-methyltransferase (COMT)	Inhibits COMT and thereby decreases the level of breakdown of Levo-DOPA outside the CNS [186].
Opicapone	COMT	Inhibits COMT and increases the level of active dopamine in the brain [187].

Tolcapone	COMT	Inhibits COMT in the periphery and CNS, which increases the level of active dopamine in the brain [188].
Selegiline	Monoamine oxidase type B (MAO-B)	Glia cells clear dopamine by oxidation by MAO-B [118]. Selegiline inhibits MAO-B and increases the level of available dopamine in the synapses [189].
Safinamide	MAO-B	Safinamide has multiple modes of actions including inhibition of MAO-B, sodium channels, and inhibition of glutamate release [190].
Amantadine	*N*-methyl-*D*-aspartate receptor	Inhibits glutamate *N*-methyl-*D*-aspartate receptors in the striatum and thereby reduces the level of dyskinesia [191].
Pimavanserin	Serotonin $5HT_{2A}$ receptor	It is a $5HT_{2A}$ reverse agonist, which decreases psychotic symptoms in PD [192].
Rivastigmine	Acetylcholinesterase (AChE)	Inhibits AChE and thereby increases the level of acetylcholine in the CNS [193]. It is approved for the treatment of mild to moderate dementia in PD and Alzheimer's' Disease [193].

1.5. MULTIPLE SCLEROSIS

MS is a progressive demyelinating neurodegenerative CNS disease and is traditionally classified as a chronic inflammatory disease [194]. MS is characterized by demyelination of the white matter in the brain and spinal cord, destruction and loss of oligodendrocytes, degeneration of axons, gliosis, inflammatory plaques, and disruption of the BBB [195]. The clinical presentation and progression of the disease are characterized by large heterogeneity [194]. Major symptoms include sensory loss, gait disability, vision loss, fatigue, impaired cognition, bladder, and bowel dysfunction [196].

The prevalence of MS is estimated to be 50–300 per 100.000 people and the incidence in Europe is estimated to 4.3 cases per 100.000 per year [194,197]. It primarily affects Caucasian women in their early adult life with a female to male ratio of 2.3 to 1 [196,197]. MS diagnosis requires dissemination of the disease in time and space and is based on the McDonald criteria [198]. Thus, diagnosis is based on clinical findings, lesions identified on MRI scans, and the presence of oligoclonal bands in the CSF [198]. MS is divided into four subtypes based on the clinical presentation [199]. Most patients experience reversible episodes of neurological deficits (attacks) in days to weeks, which are reversed to some degree over time, and then followed by a new attack [199]. This form is known as relapse-remitting MS (RRMS). A major part of the RRMS patients, over time, experiences decreased attacks followed by a progressive decline of neurological function, which is known as secondary-progressive MS (SPMS) [199]. A small proportion of MS patients experience no attacks but have a progressive decline of neurological function over years, which is defined as primary-progressive MS (PPMS) [199]. Few patients are characterized by a progressive disease including attacks, known as primary-relapsing MS (PRMS) [199].

The aetiology of MS is still not understood but a variety of risk factors is established. Major environmental risk factors include vitamin D deficiency, smoking, Epstein Bar virus, and obesity in adolescence [194]. Genetics play a role in MS, as monozygotic twins have an increased disease rate (20-30%) compared to dizygotic twins (2-5%) [200]. However, no monogenic forms of MS have been described to date, and thus MS is considered a polygenic disease [200]. People with HLA DRB1*15:01 alleles are 3 times more likely to develop MS compared to people without [194]. Importantly, the *HLA* gene and environmental risk factors interact to increase the odds ratio for developing MS [199]. E.g. smokers with the HLA DRB1*15 allele, and without the HLA-A*02 allele have 13 times higher risk of developing MS compared to nonsmoking people [200]. Recent genome-wide association studies have identified genes, which are associated with increased risk of developing MS including *IL2RA*, *IL17RA*, *CD58*, *TYK2*, *TNFRSF1A* and *STAT3* [194,200]. As of today, more than 200 loci are associated with MS [200].

Multiple hypotheses describing how MS potential develops are established. In the *intrinsic* CNS hypothesis, an initial event takes place inside the CNS causing the

release of CNS antigens [194]. These are then transported to the periphery by the lymph system or by antigen-presenting cells (APC) [194]. In the periphery, the antigens are presented to T-cells and B-cells causing an adaptive immune response against the autoantigens [194]. Lymphocytes with autoreactive properties are part of the normal lymphocyte repertoire [194]. In the *extrinsic* CNS hypothesis, the initial event, such as systemic infection, occurs outside the CNS, which leads to an irregular immune response against the CNS caused by molecular mimicry or bystander activation [33,194]. In the relapsing phases of MS, the immune system plays a central role, including T-cells, B-cells, innate immune cells, and CNS resident microglia. However, in most cases, the frequency of attacks decreases, and the disease progresses into a phase characterized by neuronal and axonal loss leading to persistent neurological deficits [199]. A variety of mechanisms is associated with the development and progression of MS including inflammation, oxidative stress, mitochondrial dysfunction, ion channel dysfunction, disruption of the HPA-axis, and alternations in the gut microbiota (**Table 6**).

Table 6: Overview of pathogenic mechanisms implicated in MS, with examples.

Mechanism	**Findings in MS**
Inflammation	Increased levels of autoreactive CD4 positive Th1- and Th17-cells in MS patients in white matter lesions [33]. Infiltration of T-cells and B-cells correlate with demyelination in RRMS [201]. Additionally, innate macrophages and microglia containing myelin debris are found in the CNS of MS patients [201]. SPMS is characterized by the infiltration of plasma cells [201]. Increased frequency of CD8 positive T-cells in grey matter cortical lesions compared to CD4 positive Th1-cells [33]. MS patients have significantly higher levels of inflammatory cytokines in both CSF and serum compared to healthy controls [202]. *In vivo* models mimicking MS are characterized by increased infiltration of immune cells in the CNS [203] and the presence of autoantibodies towards myelin proteins [204].
Oxidative stress	ROS and reactive nitrogen species are synthesized by microglia and macrophages in MS lesions and *in vivo* models of MS [205,206]. Oxidized phospholipids and DNA damage are present in active MS lesions [207]. NOX2 is upregulated in active and slowly expanding lesions in MS patients and knockout of a NOX2 subunit results in resistance to induction of MS-like disease *in vivo* [208,209]. In addition, *Nox2* is upregulated *in vivo* models of MS [210]. The antioxidant enzyme HO1 is increased in MS lesions [211] and *in vivo* models mimicking MS [212] and knockout of the

	transcription factor NRF2, which regulates HO1, results in exacerbation of disease *in vivo* [213].
Mitochondrial dysfunction	MS patients have reduced expression and activity of mitochondrial complex 1 and 3 in the cortex [214]. Brains from SPMS patients show an accumulation of deletions in mitochondrial DNA compared to age-matched controls, resulting in impaired respiration [215]. Mitochondrial pathology precedes demyelination and axonal loss *in vivo* [206,216]. Additionally, cyclophilin D can trigger the mitochondrial permeability transition, which results in disruption of all mitochondrial functions, followed by necrosis [28]. Mice with a knockout of cyclophilin D have decreased MS-like disease activity, but no changes in inflammation [217].
Ion channel dysfunction	Disruption of energy homeostasis and demyelination results in overstimulation, misallocation, and dysfunction of multiple ion channels [28]. This results in mitochondrial dysfunction, stimulation of depredating enzymes, and disrupted axonal transport, primarily due to calcium overload [28]. Disruption of normal function of sodium [218], calcium [219,220], potassium [221,222] and other ion channels are reported in MS patients and *in vivo* [28].
HPA-axis disruption	RRMS patients have increased cortisol awakening response, which is associated with disability progression based on Expanded Disability Status Scale [223]. A meta-analysis has found that there is an association between stressfull life events and worsening of disease [224]. Chronic mild stress results in exacerbation of clinical symptoms in an MS animal model [225]. Pre-exposure to chronic variable stress to female C57Bl/6J mice results in a more severe MS-like disease progression *in vivo* and higher production of pro-inflammatory cytokines in splenocytes [226].
Gut microbiota	MS patients have multiple changes in their gut microbiome, which is correlated with inflammatory gene expression in T-cells and monocytes [227]. Additionally, the different types of MS have different microbiome changes [228]. Commensal gut microbiota is required for the development of disease in the spontaneous relapse-remitting mouse model mimicking MS-like disease and is essential for the activation of autoantibody-producing B-cells [229]. Fecal transplants from monozygotic twins with MS induce a more severe disease phenotype in a spontaneous relapse-remitting mouse model compared to fecal transplants from healthy

	monozygotic twins that were associated with lower production of IL-10 from immune cells [230]. Changes in the gut microbiome and the association with inflammation in MS patients and *in vivo* models mimicking MS are reviewed in [231].

MS patients have impaired glucose metabolism in the CNS based on 18-FDG PET scans compared to healthy controls [232], which is associated with memory impairment [233]. This is consistent with increased levels of pyruvate in serum and CSF from MS patients [234]. Interestingly, rat neurons exposed to CSF from MS patients *in vitro* results in downregulation of glucose metabolism associated genes, and neuronal death [235]. MS patients have decreased levels of lipids in myelin from white matter compared to healthy controls, including long-chain FAs [236]. MS lesions also show changes in lipid composition depending on the duration of disease [237]. Accordingly, CPT1 expression is significantly upregulated in demyelinated spinal cord lesions from MS patients compared to normal-appearing white matter [238]. Additionally, MS patients have multiple changes in FA composition and levels in serum [239,240], which also is associated with increased autoantibodies with affinity to lipids [241]. Overall, this indicates that a disrupted glucose metabolism could play a central part in the development, and possibly progression of MS.

Treatments for MS focuses on modulation of the inflammatory response (**Table 7**), and are efficient in reducing relapse rates [242]. However, despite the reduction in relapse rates and inflammatory activity the majority of patients still enter the progressive phase of the disease [242–244]. The treatments have little to no effect on the development of brain atrophy [194], making neurological disability inevitably [245]. As neuroinflammation is the primary target, there is 13 FDA approved treatments for RRMS but only one for PPMS [199]. The treatment of MS is divided into an escalation, and a more aggressive induction strategy, depending on the patient's disease activity and risk factors [194]. The first-line treatment of MS has low efficacy in reducing relapses, but the more effective drugs have severe adverse events such as infections, progressive multifocal leukoencephalopathy, liver toxicity, and autoimmune reactions (schematically reviewed in [194]). In general, all treatments for MS are associated with adverse events such as influenza-like symptoms and injection complications. Therefore, there is a need for treatments that target other pathways than neuroinflammation, especially concerning the progressive phases of the disease.

Table 7: FDA approved drugs for the treatment of MS.

Drug	**Target**	**Mode of action**
Interferon-β*	Heterodimeric type I interferon receptor [246].	Reduction in antigen presentation, T cell proliferation, modulates cytokine profile, and reinstate suppressive

		immune functions [194]. Results in a 32% reduction of relapses [194].
Glatiramer acetate	Hypothesized to target antigen-presenting cells [247].	Modulates T cell differentiation, which results in the proliferation of Th2 anti-inflammatory T-cells [247]. Results in a 29% reduction of relapses [194].
Dimethyl fumarate	Targets are not completely identified but are indicated to be the master regulator of antioxidant defense, Nrf2 [248]. However, it is unclear whether the compound penetrates the BBB, and thus another target could be modulation of T-cells in the periphery [248].	Results in a reduction of the level of pro-inflammatory cytokines by shifting T-cells and B-cells towards anti-inflammatory phenotypes [249]. Moreover, dimethyl fumarate is thought to increase antioxidants in the CNS such as HO1 [249]. Results in a 51% reduction of relapses [194].
Teriflunomide	Targets dihydroorotate dehydrogenase in the pyrimidine biosynthetic pathway, which results in immunosuppression [250].	Inhibits the proliferation of B- and T-cells with autoimmune reactivity, which results in a shift towards an anti-inflammatory profile [251]. Results in a 35% reduction of relapses [194].
Fingolimod	Targets sphingosine 1-phosphate receptors by antagonistic mechanisms [252].	Inhibits lymphocytes to transit from secondary lymphoid tissue and into the circulation, which results in the dampening of inflammation [253]. Results in a 52% reduction of relapses [194].
Natalizumab	Blocks integrin α4β1 receptor [254].	The blocking of the integrin receptor results in diminished migration of T-cells and natural killer cells into the CNS [255]. Results in a 68% reduction of relapses [194].

Alemtuzumab	CD52 surface antigen on lymphocytes and monocytes [194].	Monoclonal antibody antagonizing CD52 [256], which results in depletion of T and B-cells [257]. Results in a 52% reduction of relapses [194].
Ocrelizumab	CD20 surface antigen on B cells [258,259].	Monoclonal antibody antagonizing CD20, resulting in depletion of subtypes of B-cells [260]. Reduces relapse rate by 47% [194].
Cladribine	Is a synthetic chlorinated deoxyadenosine analog [261].	Cladribine interfere with DNA synthesis and repair, which results in DNA strand breaks and thus diminishes the amount of circulating T- and B-cells [261].

* Interferon-β therapies exist in multiple forms [194].

1.6. MODULATION OF THE CPT1 SYSTEM

In section *1.3*, *1.4* and *1.5*, the presented literature indicates that dysregulated metabolism and CPT1 play a pivotal role in the described neurodegenerative diseases. Therefore, this section will first describe how lipid metabolism could affect some of the disease mechanism described in **Table 1** and *vice versa* followed by a description of how the lipid metabolism via the CPT1 system can be modulated.

1.6.1. LIPID METABOLISM AND DISEASE MECHANISMS IN NEURODEGENERATIVE DISEASES

Oxidative stress plays a major role in neurodegenerative diseases like ALS, PD and MS (**Table 2, 4, 6**) and lipids are known to be a primary target of oxidative stress [262]. Additionally, upregulated CPT1 activity are associated with increased reactive oxygen species (ROS) production in the brain *in vivo* [15]. High levels of glucose (hyperglycaemia) results in a shift from glucose metabolism towards lipid metabolism by CPT1 upregulation *in vitro* [262]. This metabolic shift results in lipid peroxidation by production of malondialdehyde [262]. Additionally, hyperglycaemia results in the production of hydrogen peroxide and this is exacerbated by addition of free FAs [262]. Overall, this indicate that lipid metabolism and oxidative stress is associated.

Inflammation and activation of the immune system play a central role in neurodegenerative disease like ALS, PD and MS (**Table 2, 4, 6**). Lipids are essential regulators of the polarization of the immune system [263]. The primary metabolic fuel depends on the type of immune cells [263]. As an example; when a pathogen invades and the inflammatory response is initiated T effector cells primarily depend on

glucose metabolism [264]. However, following the resolution of the inflammatory response the metabolic state switches towards lipid metabolism, which favours memory T cells [264]. Additionally, downregulation of lipid metabolism in conventional and plasmacytoid dendritic cells results in diminished ability to activate immune cells by co-stimulatory molecules, suppress the production of pro-inflammatory cytokines and chemokines [265]. Further, downregulation of CPT1 lipid metabolism in bone marrow derived macrophages attenuate inflammatory activity [266]. Increased levels of oxidized lipids stimulates inflammatory responses in macrophages [267]. Based on this it seems possible that lipid metabolism and inflammation is associated.

Mitochondrial dysfunction is implicated to be an essential component in ALS, PD, MS (**Table 2, 4, 6**) and other neurodegenerative diseases. As presented above the mitochondria play a central role in the metabolism of lipids. Increased mitochondrial lipid metabolism results in production of excess ROS [15], changes in pH and fragmentation of the mitochondria [268,269]. Thus linking mitochondrial function and metabolism tightly.

HPA-axis dysregulation and changes in glucocorticoids are indicated – at least to some extent – to play a role in neurodegenerative diseases (**Table 2, 4, 6**). Increased production and release of glucocorticoids results in reduced uptake of glucose, insulin resistance and lipolysis [270,271]. Cortisol treatment in healthy volunteers causes increased metabolism of long-chain FAs [36]. Additionally, patients with Cushing syndrome (due to treatment with excessive cortisol) have changes in β-oxidation [272]. Further, an increased activity of the HPA-axis, as seen in ALS, PD and MS (**Table 2, 4, 6**) and depression, have been shown to reduce the level of long-chain FAs in the blood and brain, indicating increased lipid metabolism [273]. This indicates a link between dysregulation of the glucocorticoid homoeostasis and lipid metabolism.

Pathogenic changes in myelin has been linked to MS (**Table 6**) for more than a century. However, data indicate that myelin is affected in ALS (**Table 2**), and recently possibly also PD [274,275]. The dry mass of lipids account for up to 85 % of weight of myelin in the CNS and periphery and is crucial for the long-term stability of myelin [276,277]. In this regard, long-chain FAs moieties are the most typical myelin lipids [277]. Myelin synthesis only takes around five hours when it is initiated by the oligodendrocytes, and thus demand a vast amount of lipids [276]. Based on this, it seems evident that increased lipid metabolism can have detrimental effects on myelin homeostasis [277].

The gut microbiota have a long variety of functions including modulation of the immune system and metabolism and vice versa the metabolism is known to modulate the gut microbiome [7,42,278,279]. Additionally, the CNS and gut is linked through the gut-brain-axis [42,278] therefore making it possible that changes in the gut microbiome could result in changes in the metabolism affecting the CNS and periphery.

Based on the description above it seems possible that lipid metabolism could be associated with multiple disease mechanisms and therefore it seems highly relevant to review how the CPT1 lipid metabolism can be modulated (e.g. by pharmacological and genetic modulation). This will be the focus of the next two sections.

1.6.2. PHARMACOLOGICAL DOWNREGULATION OF CPT1

Multiple pharmacological CPT1 and β-oxidation inhibitors exist including, but are not limited to, perhexiline [280], etomoxir [281] and ranolazine [282]. In *manuscript I, II, IV*, and *V* etomoxir was used, and thus this compound is the focus of this paragraph. Etomoxir is a small molecule drug and acts as an irreversible CPT1 antagonist with an affinity for both CPT1A and CPT1B [283]. Etomoxir exists in several syntheses forms described in the patent literature, including the form used in the manuscripts included in this thesis [283]. As a result, many biochemical properties, including pharmacokinetics and dynamics are not presented here. Etomoxir has a molecular weight of 320 – 330 Da, and exists in multiple forms, including an etomoxir-ethyl ester, which is soluble in oil (lipophilic) at approximately 37 °C. These chemical properties make it favourable for diffusion across the BBB [284]. Etomoxir was originally developed for the treatment of non-insulin depend type 2 diabetes by Byk Gulden Pharmaceuticals, Germany [285]. Additionally, etomoxir was investigated for the treatment of congestive heart failure by Medigene AG, Germany. However, the clinical trial was terminated prematurely due to increased liver transaminase levels in four patients [286]. These adverse events could be explained *in vitro* by interactions between the β-blocker metoprolol and etomoxir (*data not published)* because metoprolol downregulates glucose metabolism [287]. Etomoxir downregulates β-oxidation and increases glucose utilization *in vitro, in vivo*, and in humans [281,288] and upregulates PPARα [283]. Therefore, etomoxir is a potential candidate to evaluate the effect of downregulating CPT1 in neurodegenerative diseases.

1.6.3. GENETIC *CPT1A* DOWNREGULATION BASED ON HUMAN MUTATIONS

Multiple human mutations in the *CPT1A* gene are reported [289]. One of the most prevalent *CPT1A* mutations is a missense mutation at position 1436 C to T causing a substitution of proline for leucine at codon 479 (P479L) [290]. This is found at extremely high rates in Canadian and Greenland Inuit but also in Northeast Siberian [290,291]. The P479L mutation leads to a 78 % reduction in protein activity compared to non-carriers, which results in a 22 % residual activity [292]. The mutation is associated with an increased risk of hypoketotic hypoglycemia, infant mortality, and infections [292,293]. Interestingly, the P479L mutation is associated with changes in HDL lipoproteins and composition of PUFAs and monounsaturated FAs [294,295]. The artic Inuit population

has a lower prevalence of people suffering from MS [296,297] and ALS [298] compared to the background population. Overall, this indicates that there might be causality between the activity of CPT1 and neurodegeneration.

A shift in metabolic pathways seems as a common theme in the pathology of neurodegenerative diseases such as ALS, PD and MS, but not limited to these. Therefore, a relevant question is how this can be investigated? In *manuscript I – V in vivo* models were used, and therefore the next section will shortly review some general concepts of modelling.

1.7. MODELING OF NEURODEGENERATIVE DISEASES

In accordance to the Committee on New and Emerging Models in Biomedical and Behavioral Research, USA, a biomedical model can be defined as "*a surrogate for a human being, or a human biologic system, that can be used to understand normal and abnormal function from gene to phenotype and to provide a basis for preventive or therapeutic intervention in human diseases.*" [299].

Neurodegenerative diseases such as ALS, PD and MS can be modelled *in vitro* using e.g. cell cultures or *in vivo* using e.g. animals. The disease can be modelled by chemical/environmental toxins or by genetic manipulation both *in vitro* and *in vivo*. Benefits of *in vitro* modelling includes lower costs and durability of studies [300]. In addition, the human induced pluripotent stem cells provide a novel platform because they can be derived from patients with specific genetics and diseases, which makes it possible to study cellular pathological changes and dynamics in humans [300]. Neurons and glial cells can be cultivated together in different well-systems, but this does not include a BBB, which is an essential component, protecting the CNS from the periphery [300]. Further, *in vitro* models lack complex neuronal circuits and absence from vascular and immunologic components [301]. Therefore, *in vitro* studies can only examine molecular changes and evaluate mode-of-actions of drugs in a local system but not evaluate molecules and drugs abilities to cross the BBB. In addition, *in vitro* models cannot investigate interactions between multiple system such as the CNS, muscles and the gut.

However, no animal model fully recapitulates all phenotypic features of human neurodegenerative disease [301]. Nevertheless, many recapitulate some of the pathological and clinical features of the human disease. A benefit of using *in vivo* modelling is the possibility to investigate interactions between multiple systems, obtain different biological fluids during a study, and investigate physiological effects longitudinal making it possible to evaluate surrogate markers mimicking clinical symptoms in humans [301]. However, the anatomy of rodents have many differences compared to humans, especially considering the development of the brain and CNS [301]. Additionally, the lifespan of rodents are short (1 – 2 years) compared to humans

[301]. This is a problem in the translation of neurodegenerative diseases, as age is a major risk factor for developing neurodegenerative disorders in humans [119]. In addition, many *in vivo* models are based on inbreed animals to control for genetic factors, but this does not reflect genetic diversity as seen in humans. These, and several other factors such as differences in time point for treatment initiation, account for the problems with translating drug-candidates from pre-clinical studies and into approved therapies for humans. However, despite these problems, animal models have resulted in increased understanding of the molecular, cellular and multisystem interactions in neurodegenerative diseases [301]. In addition, they provide a valuable platform for investigating possible targets in neurodegenerative diseases with the limitations kept in mind.

Based on the above description the following chapters will include a description of testing the effect of modulating CPT1 or CPT1A activity in *in vivo* models mimicking ALS, PD and MS.

CHAPTER 2. OBJECTIVES

Neurodegenerative diseases such as ALS, PD and MS affect millions of people worldwide, which makes it essential to elucidate the underlying aetiologies in order to develop potential novel targets for treatments due to the current lack of effective therapies. At present, it is evident that metabolism is dysregulated in neurodegenerative diseases especially that glucose metabolism is downregulated and lipid metabolism upregulated in ALS, PD and MS, as presented in chapter 1. However, the effects of downregulating CPT1 mediated lipid metabolism to restore metabolic homeostasis and ameliorate pathogenic mechanisms in *in vivo* models mimicking these neurodegenerative diseases remains unexplored and thus a knowledge gap exists. Therefore, the overall *aim* of this PhD thesis has been to investigate the role of dysregulated metabolism, and to evaluate the effects of targeting CPT1 (by etomoxir and *Cpt1a P479L* mutations) in rodent models mimicking ALS, PD and MS (**Figure 2**). In general, we *hypothesized* that downregulation of CPT1 regulated lipid metabolism would ameliorate or delay progression of clinical disease symptoms in these models in connection with attenuated disease mechanisms such as inflammation, oxidative stress, mitochondrial biogenesis and demyelination whereas upregulation of CPT1 activity (by 60 % high fat diet (HFD) or corticosterone (CORT) administration) would result in exacerbation of the clinical disease as well as the disease mechanisms.

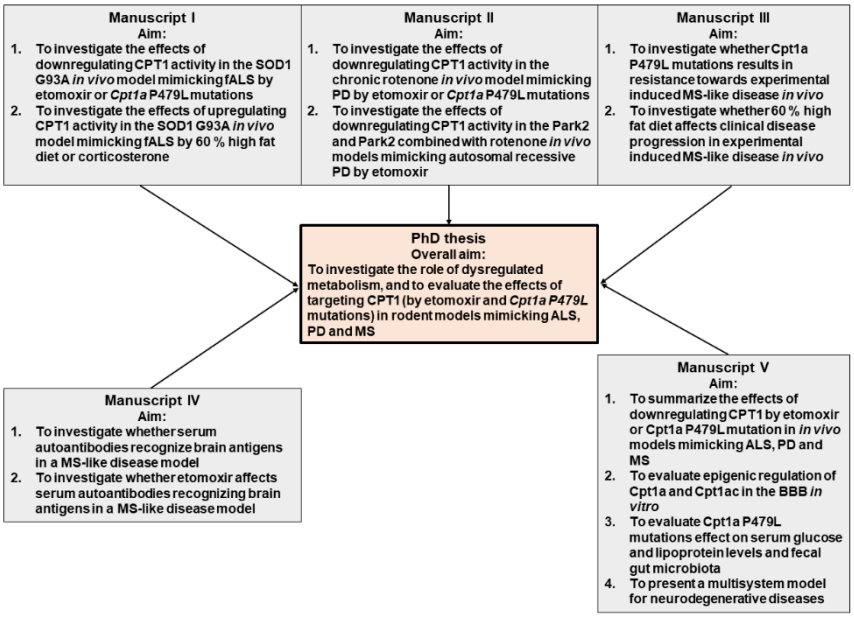

Figure 2: **Aims of PhD thesis and manuscript I-V.** Diagram illustrating how the aims of the manuscripts are connected to the overall aim of the PhD thesis

To investigate the overall aim of the PhD thesis five studies, with the following aims, were conducted:

2.1. MANUSCRIPT I

Aims: To investigate the effects of pharmacological downregulating CPT1 by etomoxir, and genetically by the *Cpt1a* P479L mutation using the SOD1 G93A mouse model. Additionally, to assess the effects of 60% HFD and CORT on establishment and disease progression in the SOD1 G93A mouse model. The effects were based on motor and non-motor behaviour symptoms, inflammatory, oxidative stress, metabolic, denervation and atrophy markers. Finally, to investigate the effects of etomoxir, *Cpt1a* P479L mutation, HFD and CORT on the fecal gut microbiome in the SOD1 G93A model using 16s ribosomal ribonucleic acid (rRNA) sequencing.

Hypothesis: Downregulation of CPT1 by etomoxir and CPT1A by *Cpt1a* P479L mutation results in slower disease progression and attenuation of inflammation, oxidative stress, denervation and atrophy and a shift towards glucose metabolism in the SOD1 G93A mouse model mimicking fALS. Upregulation of CPT1 by HFD or CORT results in exacerbation of clinical disease progression, inflammation, oxidative stress, denervation and atrophy and decreased glucose metabolism in the SOD1 G93A mouse model mimicking fALS.

2.2. MANUSCRIPT II

Aims: To investigate the effects of pharmacological downregulating CPT1 by etomoxir, and genetically by the *Cpt1a* P479L mutation using the chronic rotenone mouse model mimicking some aspects of PD-like disease. Additionally, to assess the effects of downregulating CPT1 by etomoxir in the *Park2* exon 3 deletion model mimicking some aspects of early-onset autosomal recessive PD. Further, to investigate the effect of etomoxir in a *Park2* mutation model combined with chronic rotenone model. The effects were based on motor and non-motor behaviour symptoms, inflammatory, oxidative stress, metabolic markers, tyrosine hydroxylase, α-syn, and dopamine levels. Finally, to investigate the effects of etomoxir and *Cpt1a* P479L mutation in these disease models on the fecal gut microbiome using 16s rRNA sequencing.

Hypothesis: Downregulation of CPT1 by etomoxir and CPT1A by *Cpt1a* P479L mutation results in amelioration of motor and non-motor behaviour symptoms in the chronic rotenone mouse model and resistance towards induction of disease by rotenone, respectively. Etomoxir and *Cpt1a* P479L mutation results in decreased death of dopaminergic neurons, levels of α-syn and increased dopamine levels in the midbrain. Downregulation of CPT1 by etomoxir results in amelioration of motor and non-motor behaviour symptoms, increased glucose metabolism and decreased

inflammation and oxidative stress in the *Park2* mouse model. Downregulation of CPT1 by etomoxir results in decreased symptoms, increased glucose metabolism, decreased death of dopaminergic neurons, decreased levels of α-syn and increased levels of dopamine in the midbrain in a combined *Park2*-rotenone mouse model.

2.3. MANUSCRIPT III:

Aims: To establish an experimental autoimmune encephalomyelitis (EAE) mouse model and evaluate the effect of the *Cpt1a* P479L mutation on the disease induction and progression. Additionally, to evaluate the effects on myelin basic protein (MBP) and CPT1A expression in the CNS, and oxidative stress gene expression. Finally, to investigate the effect of 60% HFD on disease progression.

Hypothesis: Cpt1a P479L mutated mice show resistance towards induction with myelin oligodendrocyte glycoprotein (MOG) induced EAE with decreased clinical symptoms, decreased demyelination and oxidative stress markers. 60% HFD results in exacerbation of disease progression and motor impairment in wild type mice induced with EAE mimicking MS-like disease.

2.4. MANUSCRIPT IV

Aims: To investigate the effect of etomoxir and interferon-β on the autoantibody-antigen response in a MBP induced EAE rat model in order to establish potential biomarkers, and evaluate the treatments effect on the B-cell response.

Hypothesis: Rats induced with EAE have increased serum autoantibodies recognizing brain antigens, which is decreased by etomoxir and to a lesser degree by interferon-β.

2.5. MANUSCRIPT V

Aims: To summarize the findings of the effect of etomoxir and *Cpt1a* P479L mutation in multiple *in vivo* models mimicking ALS, PD, and MS. To evaluate the effect of *Cpt1a* P479L mutation on serum lipoproteins and glucose levels. Additionally, to investigate the role of epigenetic regulation of *Cpt1a* and *Cpt1c* expression in astrocytes, pericytes and endothelial cells from the rat BBB. Further, to assess changes in the fecal gut microbiome in *Cpt1a* P479L mutated mice. Finally, to present a systemic framework for the understanding of how the glucose-lipid metabolism balance is involved in the different processes that are key in the development and progression of neurodegenerative diseases.

Hypothesis: Downregulation of CPT1 activity by etomoxir or *Cpt1a* P479L mutation results in amelioration of disease in multiple *in vivo* models mimicking ALS, PD and

MS. *Cpt1a* P479L mutation results in lower serum levels of glucose, LDL, decreased LDL/HDL ratio and changes in the composition of the fecal gut microbiome. In addition, *Cpt1a* and *Cpt1a* is epigenic regulated in the BBB *in vitro*. Finally, *manuscript V* hypothesize that the development and progression of neurodegenerative diseases can be explained from a multisystem perspective.

CHAPTER 3. METHODOLOGICAL CONSIDERATIONS

During my PhD I have established and conducted studies using multiple *in vivo* models. We chose to use *in vivo* models to study the systemic interaction between multiple organ systems, which is not possible *in vitro*. Furthermore, a large array of genetic manipulated *in vivo* models exists. Therefore, this chapter will first describe legal aspects and relevant guidelines for the reporting of *in vivo* studies followed by a description of the choice of animal models to investigate the role of dysregulated metabolism, and how to evaluate the effects of targeting CPT1 in models mimicking some aspects of ALS, PD and MS. Finally, the chapter will present data examining interrater variation in the analysis of motor and non-motor behaviour parameters.

3.1. LEGAL ASPECTS, THE $3R_S$ AND ARRIVE GUIDELINES

The use of animals in scientific experiments is regulated by national and international legislation. All animal experiments were approved by the Danish Animal Experiments Inspectorate and conducted according to their guidelines. The legislation is based on the 3Rs (replacement, reduction and refinement). Replacement deals with methods how to avoid the use of animals such as *in vitro* or human participants, reduction deals with methods how to minimize the number of animals and refinement deals with methods how to minimize suffering and increase animal welfare [302]. As described in *section 1.7* multiple organ systems cannot be modelled *in vitro* and therefore replacement was not possibly in the manuscripts included in this thesis. However, the studies were appropriately designed, analysed and reproduced, if possible, to adherer to the reduction criteria. In addition, all interventions were done in the most humane manner and humane endpoints were pre-established and approved to adherer to the refinement criteria.

At the core of science lies reliability and reproducibility, but multiple *in vivo* studies have been impossible to reproduce due to vague reporting. Therefore, to heighten the reporting in animal studies, the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines have been established and was updated in July 2020 [303]. In accordance with these guidelines all *in vivo* studies have to report: "*study design, sample size, inclusion and exclusion criteria, randomisation, blinding, outcome measures, statistical methods, experimental animals, experimental procedures and results*" [303]. All the *in vivo* studies in *manuscript I - V* were based on the ARRIVE guidelines. Animals were randomized into interventions groups following breeding or following arrival. The clinical behaviour parameters, which is described in the following sections, were evaluated in a random manner. In addition, the experimenters

were blinded to genotypes and treatment groups during data collection and analysis of video-recorded clinical parameters.

3.2. SOD1 G93A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

Manuscript I and *V* evaluates the effects of downregulating CPT1 activity, the effects of stimulating lipid metabolism through a 60% HFD and the effects of increased levels of CORT using the SOD1 G93A mouse model. The SOD1 G93A mutation was the first mutation linked to autosomal dominant fALS in 1993 by Rosen et al. [52]. The SOD1 G93A mouse model was established in 1994 by Gurney et al [53]. The SOD1 G93A mouse model have approximately 18 - 20 copies of the human *SOD1* gene with G93A mutation at chromosome 12 [53]. The development of the SOD1 G93A model has resulted in SOD1 G93A mouse models on different genetic backgrounds [304]. In the studies presented in this thesis, we used the SOD1 G93A mouse model based on the C57Bl/6J genetic background (hereafter SOD1 G93A). This background was chosen because the original C57BL/6J × SJL/J (B6-SJL) background have a more severe clinical disease progression [305] and males and females have significant different survival on the B6-SJL background, which is not the case for the C57Bl/6J background [306]. Due to multiple cohorts, and interventions, we had to use a substantial amount of transgenic SOD1 G93A mice. Transgenic SOD1 G93A female mice are poor breeders and thus transgenic SOD1 G93A males had to be used to maintain the colony at our animal facility. Therefore, only female mice was used in the studies presented in *manuscript I* and *V*. Fortunately, a meta-analysis show no difference in disease onset and survival between B6.SOD1 G93A females and males [307]. This is consistent with data obtained from our animal facility (**Figure 3**). Furthermore, sALS affects men more frequently than women, but fALS affects men and women equally [47].

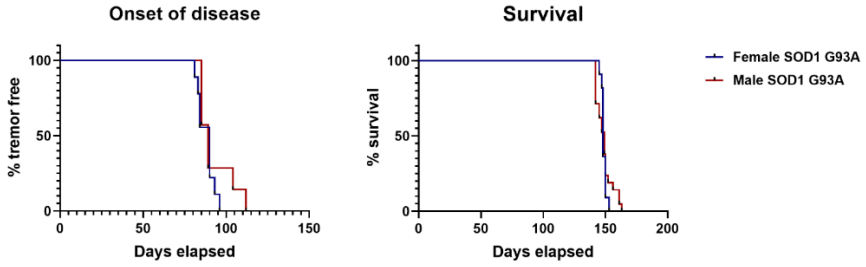

Figure 3: **Difference in disease onset and survival time between female and male SOD1 G93A mice.** Log-rank (Mantel-Cox) test showed no significant difference ($p = 0.35$) in disease onset between female (median disease onset = 90 days, n = 9) compared to male SOD1 G93A mice (median disease onset 89 days, n = 7). Disease onset was defined as the presence of fine-tremor in the hind legs. Log-rank (Mantel-Cox) test showed no significant difference ($p = 0.67$)

in survival between female (median survival = 148 days, n = 11) compared to male SOD1 G93A mice (median survival 149 days, n = 21).

The transgenic SOD1 G93A mice present with a variety of symptoms including neurological disability, weight loss, muscle dysfunction and cognitive decline. Based on this we applied several motor and non-motor behavioural tests to evaluate the interventions' effect on the clinical disease progression (**Table 8**). All motor tests can discriminate between transgenic SOD1 G93A and non-transgenic mice from an early age (**Figure 4**).

Table 8: Clinical-relevant behavioural tests applied in the SOD1 G93A mouse model.

Test	**Function evaluated**	**Description**
Neurological disease score	General neurologic disability	Mice are visual inspected by a minimum of one blinded experimenter and given a score between 0 and 4 [308]. In our animal facility we were only allowed to conduct the studies until a score of 4. Neuroscore 0 = normal position of hindlimbs, full extension of hindlimbs when suspended by its tail for a minimum of 2 s, normal walking and righting reflex. Neuroscore 1 = tremor in hindlimbs, normal extension, walking and righting reflex. Neuroscore 2 = Tremor in hindlimbs, no full extension of hindlimbs when suspended by its tail, normal walking and righting reflex. Neuroscore 3 = Tremor in hindlimbs, no full extension of hindlimbs, wobbling gait, normal righting reflex. Neuroscore 4 = Tremor, no extension of hindlimbs, difficulty with walking, paralysis of one of both hindlimbs, normal righting reflex. Neuroscore 5 = unable to get turn around within 30s when placed on its side. (*Manuscript I*)

Hangwire	Muscle strength and coordination	A mouse is placed on a wire-lid, gently turned upside down and the latency to fall is measured in seconds [308]. The upper and lower cut-off was set to 180 s and 5 s, respectively. The highest latency to fall is recorded. Each mouse receive 3 trials [309].
Grip strength	Muscle strength	A mouse is placed on a wire-mesh, which is attached to a grip strength meter, and subsequently pulled by its tail [279]. The maximum grip force is measured when the mice release its limbs from the wire-mesh [310]. The grip strength is measured in grams and normalized to each mouse bodyweight to account for differences in body weight [310].
Rotarod	General neurological function, motor function, balance	Mice are placed on a rotating rod, which accelerate from 4 rounds per minute to 40 rounds per minute over a period of 300s [311]. The latency to fall is noted. Each mouse receive three trials per session. Mice are habituated to the rotarod over a period of three days.
Cylinder test	Sensorimotor function	A mouse is placed in a transparent glass cylinder for three minutes and recorded by a video camera [312]. The number of rears is subsequently counted [279].
Spontaneous alternation y-maze test	Visuospatial memory	A mouse is placed in the middle of a y-shaped maze and allowed to explore the arms for five minutes [313]. The test is recorded by a video camera and analysed to evaluate the number of entries into the three arms and the spontaneous alternation percentage [313].

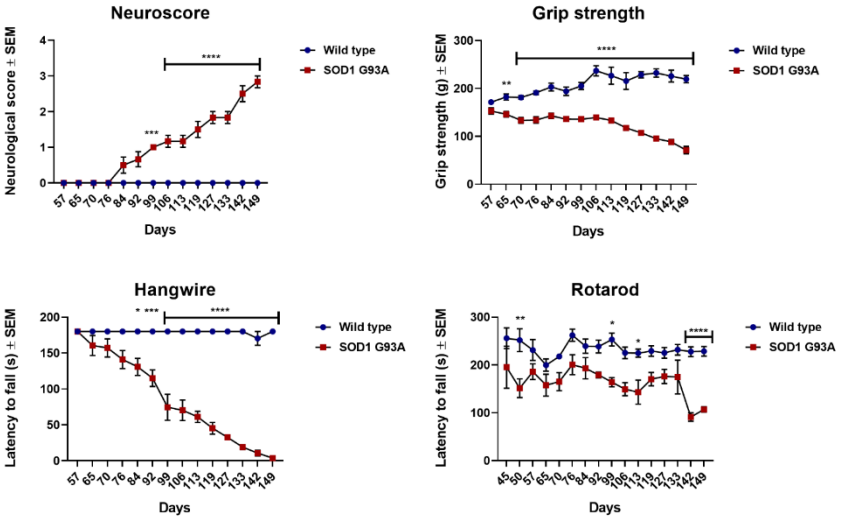

Figure 4: **SOD1 G93A mice present with motor symptoms from age 50.** Healthy wild type C57Bl/6J mice (n = 3 – 4) were compared to female SOD1 G93A mice (n = 3 – 6) from day 50 (rotarod) and day 57 (Neuroscore, grip strength and hangwire test). All motor tests were able to discriminate between the genotypes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistics: Repeated measure Two-way ANOVA with bonferroni post hoc test.

As this thesis aims to investigate the effect of targeting metabolic dysregulation in the SOD1 G93A model, it is crucial that the SOD1 G93A mouse model have metabolic disturbances. This has been illustrated by a variety of studies [107,108,78,80] and data in *manuscript I*, making this model relevant to assess the hypothesis presented in this thesis. Additionally, the SOD1 G93A mouse model is characterized by death of lower MNs, inflammation, oxidative stress, mitochondrial dysfunction, loss of myelin, gut dysbiosis, and denervation, among other pathogenic mechanisms, as described in **Table 2**, *manuscript I* and illustrated in **Figure 5**.

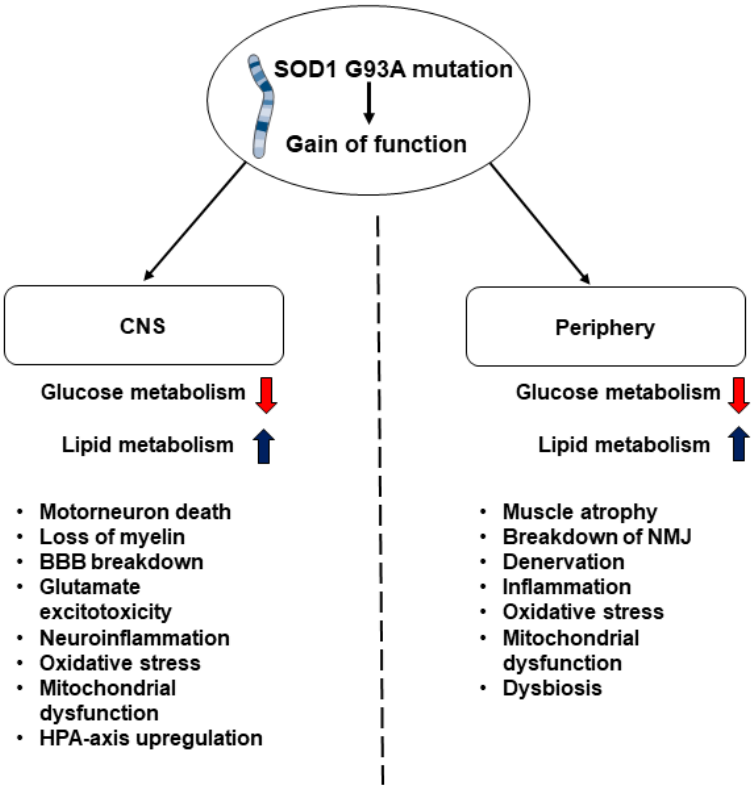

Figure 5: **The SOD1 G93A mutation results in activation of multiple pathological processes.** The SOD1 G93A mutation causes a gain of function in the SOD1 protein, which results in downregulation of glucose metabolism, upregulation of lipid metabolism and pathological characteristics within the CNS and in the periphery as described in **table 2**. Key pathogenic process are mentioned but not limited to the list. Illustration elements was obtained from Servier Medical ART with license; https://creativecommons.org/licenses/by/3.0/. No changes were made to the elements.

Following the establishment of the SOD1 G93A mouse models in 1994, multiple transgenic ALS *in vivo* models have been developed including *C9orf72*, *Fus* and *Tardp* transgenic models [314]. This enable researchers to address complex hypotheses, and to evaluate translational aspects such as treatment efficacy on different genetic subtypes of fALS and to dissect the different pathogenic mechanisms. Furthermore, a large proportion of sALS patients have mutations in genes associated with fALS, and thus transgenic models provide a crucial platform to obtain data relevant for the aetiology and pre-clinical screening of drug targets in both sALS and fALS [54].

The effects of downregulating CPT1 by etomoxir were examined as described in section 1.6.2. To validate the role of CPT1A in neurodegenerative diseases a novel *Cpt1a* P479L mouse strain, the artic variant of *Cpt1a*, which have 22 % CPT1A activity compared to the wild type protein in all tissues [210] was generated. The *Cpt1a* P479L mouse strain was based on a C57Bl/6J background [210]. The *Cpt1a* P479L mouse strain was crossed with SOD1 G93A mice to obtain SOD1 G93A mice with a heterozygote [279] and a homozygote *Cpt1a* P479L mutation to assess the effect of genetic downregulation on disease progression in the SOD1 G93A mice.

3.3. *IN VIVO* MOUSE MODELS OF PARKINSON'S DISEASE

Manuscript II and *V*, tested hypotheses using the rotenone and *PARK2* mouse models mimicking some aspects of PD-like disease. PD affects men more frequently than women [119], and based on this we used male mice. The inbreed C57Bl/6J background was chosen based on the published literature [315]. Multiple toxin-induced PD *in vivo* models exists including 6-hydroxydopamine (6-ODHA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone [135]. Rotenone is a lipophilic pesticide that blocks complex I in the mitochondria, and thereby results in mitochondrial dysfunction [174]. Its lipophilic properties result in rapid distribution in the CNS following peripheral administration [316,317]. Importantly, a case-control study found that human rotenone exposure increased the odds ratio of developing PD by 2.5 [318], underpinning the translational value of rotenone. Rotenone models in rodents have been associated with large variation in the proportion of animals that develop the disease and inconsistent PD-like pathology [319]. Nonetheless, during the last two decades a chronic rotenone mouse model that recapitulates many of the clinical [320] and pathological [321] features of PD have been developed, as illustrated in **Table 4**, *manuscript II*, **Figure 6** and **Figure 7**. Based on this, we have used a 30 mg/kg rotenone dosing regimen, as doses above 30 mg/kg results in severe toxicity and total loss of dopaminergic neurons in SN [320], whereas doses below 30 mg/kg results in a small loss of dopaminergic neurons [321] . Additionally, male rodents have shown to be more susceptible to rotenone-induced PD-like disease with higher levels of inflammation and deposition of α-syn in the SN compared to females [322]. The oral gavage method was chosen because humans are exposed to rotenone in the environment and not by subcutaneous or intravenous administration, thus providing translational value [318]. Additionally, rotenone results in downregulation of glucose metabolism and upregulated CPT1A activity *in vitro* [174] and *in vivo* [175], thereby making it a suitable model for studying the effect of pharmacological and genetically downregulation of CPT1 in PD-like disease.

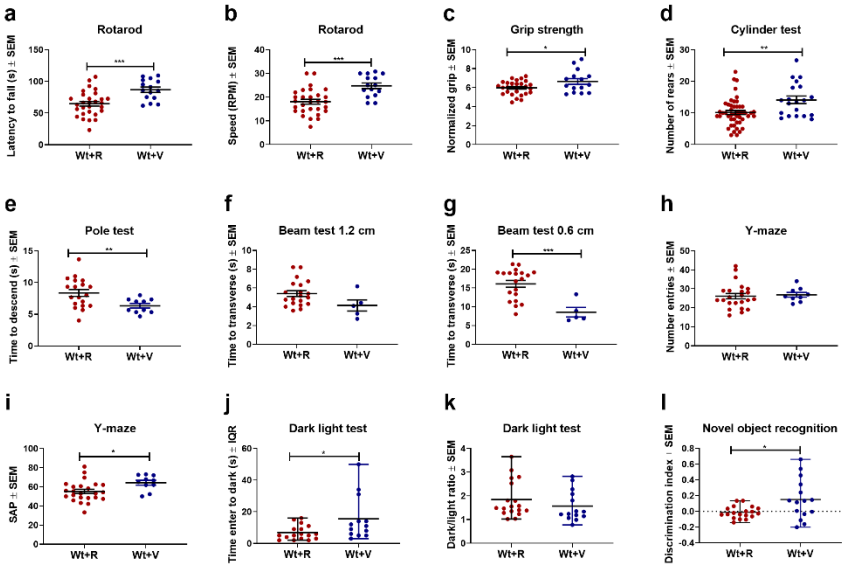

Figure 6: 32 days of 30mg/kg oral administered rotenone induces a behavioural phenotype mimicking some aspects of PD in C57Bl/6J male mice. a) Rotarod (mean latency to fall)**. b)** Rotarod (mean RPM)**. c)** Normalized grip strength**. d)** Cylinder test (mean number of rears). **e)** Pole test (mean time to descend)**. f)** Beam test 1.2 cm (mean time to transverse)**. g)** Beam test 0.6 cm (mean time to transverse)**. h)** Y-maze test (number of entries)**. i)** Y-maze (mean SAP)**. j)** Dark light box test (median time to enter dark)**. k)** Dark light box test (mean dark/light ratio)**. l)** Novel object recognition test (mean discrimination index)**.** Animals were tested at day 32. Error bars represent the standard error of the mean (SEM), or interquartile range of the median (IQR). Significant differences; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$. Wt=wild type, CMC=carboxymethylcellulose sodium salt, R=rotenone, V=vehicle, RPM=rounds per minute, SAP=spontaneous alternation percentage. Statistics: Two-tailed unpaired t-test, Mann-Whitney U test.

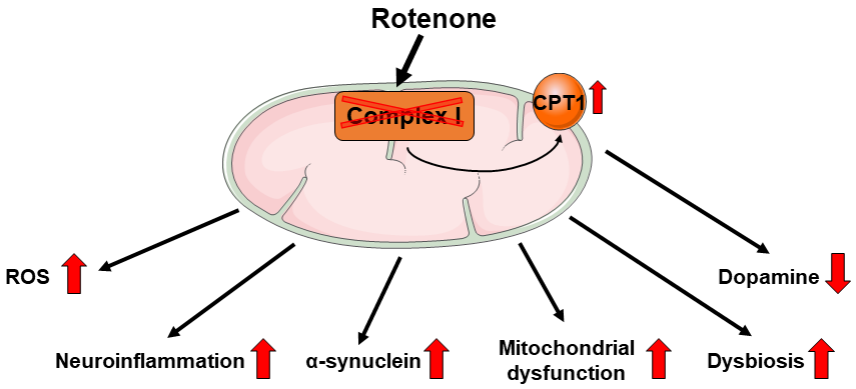

Figure 7: **Rotenone induces several pathological processes associated with PD**. Rotenone

blocks the complex I in the mitochondrion, which results in an upregulation of CPT1 and β-oxidation [174,175]. Rotenone causes production of reactive oxygen species (ROS) and oxidative stress, activation of reactive microglia and other immune cells resulting in neuroinflammation [320–322]. Further, it results in α-synuclein oligomerization, mitochondrial dysfunction and loss of dopaminergic neurons in the substantia nigra [315]. Rotenone also elicits dysbiosis, increased intestinal permeability and changes in the gut microbiota [163], among other processes. Illustration elements was obtained from Servier Medical ART with license; https://creativecommons.org/licenses/by/3.0/. No changes were made to the elements.

In addition, the transgenic B6.129S4-*Prkn*^{tm1Shn}/J (hereafter referred to as *Park2*) mouse model, which mimics an early-onset autosomal recessive form of PD [323] was used to evaluate the effects of downregulating CPT1 in a genetic form of PD. The *Park2* mice models an exon 3 deletion, which is the most common mutation in autosomal recessive PD [123]. The *PARK2* mutation is characterized by mitochondrial dysfunction [149], oxidative stress [149], disrupted glucose metabolism [324] and increased lipid metabolism [325]. Thus, providing a suitable platform to investigate the effects of modulating CPT1 in lipid metabolism in PD-like disease. However, the *Park2* mice only modulates some aspects of the human *PARK2* early-onset PD [323] and therefore we also combined rotenone with the *Park2* mutation. Besides the *Park2* mouse model a large variety of transgenic PD mouse models exists, which all modulates different elements of the clinical and pathological characteristics of PD [135,326].

In *manuscript II* and *V* a large variety of clinical behavioural test were used to evaluate whether impairment of motor and non-motor functions, mimicking some symptoms seen in PD [119], were present (**Table 9**).

Table 9: Clinical-relevant behavioural tests applied in the PD mouse models.

Test	Function evaluated	Description
Rotarod	General neurological function, motor function, balance	Mice are placed on a rotating rod, which accelerate from 5 rounds per minute to 40 rounds per minute over a period of 140s [311]. The maximum cut-off time was set to 180s. The latency to fall is noted. Each mouse receive three trials per session. Mice are habituated to the rotarod over a period of three days [311].
Grip strength	Muscle strength and coordination	A mouse is placed on a wire-mesh, which is attached to a grip strength meter, and subsequently pulled by its tail [279]. The maximum grip force is measured when the mice release its limbs from the wire-mesh

		[310]. The grip strength is measured in grams and normalized to each mouse bodyweight to account for differences in body weight [310].
Beam test	Sensorimotor function and balance	A mouse is placed on a beam, recorded by a video camera and the time to transverse is noted [312]. Each mouse receives three trials per session and is habituated to the test over a span of three days [312].
Pole test	Sensorimotor function and balance	A mouse was placed on the top of a pole with its head facing upwards, allowed to turn around and descend to the bottom [163]. The test was recorded by a video camera. Each mouse receives three trials per session and is acclimatized to the test over a span of three days [163].
Cylinder test	Sensorimotor function	A mouse is placed in a transparent glass cylinder for three minutes and recorded by a video camera [312]. The number of rears are subsequently counted [279].
Novel object recognition test (NOR)	Short term memory	A mouse is placed in a rectangular box and allowed to explore the open field for ten minutes [327]. Afterwards, the mouse is removed from the box, two identical objects are placed in the box and the mice is placed back in the box for 10 min to familiarize with the objects [327]. Finally, the one of the familiar objects are replaced by a novel object, a video camera is turned on, and the mouse is allowed to explore the two objects [327]. Following the test, the video is recorded and the time spent to explore the familiar and novel object is recorded and a discrimination index is calculated [327].
Spontaneous alternation y-maze test	Visuospatial memory	A mouse is placed in the middle of a y-shaped maze and allowed to explore the arms for five minutes [313]. The test is recorded by a video camera and analysed to evaluate the number of entries into the three

		arms and the spontaneous alternation percentage [313].
Dark light box test	Anxiety-like symptoms	A mouse is placed in a box divided into a light and dark chamber with a small opening for five minutes [328]. The mouse is initially placed in the light chamber and recorded by a video camera. The total time of transitions between the light and dark chamber, and total time spent in light versus dark is noted [328].

3.4. *IN VIVO* MOUSE MODELS OF MULTIPLE SCLEROSIS

Manuscript III, *IV* and *V* includes *in vivo* models mimicking MS. Multiple models imitating MS exists but none of them recapitulates all features of MS [329]. The state-of-the-art MS *in vivo* model is EAE, which exists in an active immunization, an adoptive transfer and a spontaneous form [329,330]. Active immunized EAE can be induced by MBP, PLP and MOG peptides, in conjunction with or without immunostimulatory cocktails like complete Freund's adjuvant with mycobacterium tuberculosis, which induces different phenotypes and pathologies (**Figure 8**). MBP accounts for up to 40% of the CNS and up to 15% of the peripheral myelin proteins and was one of the first isolated proteins that was shown to induce EAE [329]. Active immunization with MBP induces a disease phenotype distinguished by an acute paralytic attack from which the animals recover partially or completely, mimicking the clinical phases seen in RRMS [329]. SJL/J and PL/J mice are highly susceptible to MBP-induced EAE, whereas C57Bl/6J mice are resistant [329,331]. PLP is a transmembrane protein and constitutes a vital element in the compaction of myelin in the CNS [329]. Active immunization with PLP induces a disease phenotype in SJL/J mice characterized by multiple relapses and remissions [329]. MOG constitutes a small part of the myelin (up to 0.05%) and is located at the outer surface of CNS myelin [329]. However, despite the fact that MOG only constitutes a small proportion of the myelin sheet more MOG-reactive T-cells are present in MS patients than MBP- and PLP-reactive T-cells. Induction with MOG peptides results in a chronic progressive disease phenotype in C57Bl/6J mice characterized by no remission mimicking SPMS and PPMS [329,331]. MOG peptides also induce EAE in a variety of other mouse strains including SJL/J and PL/J mice [329].

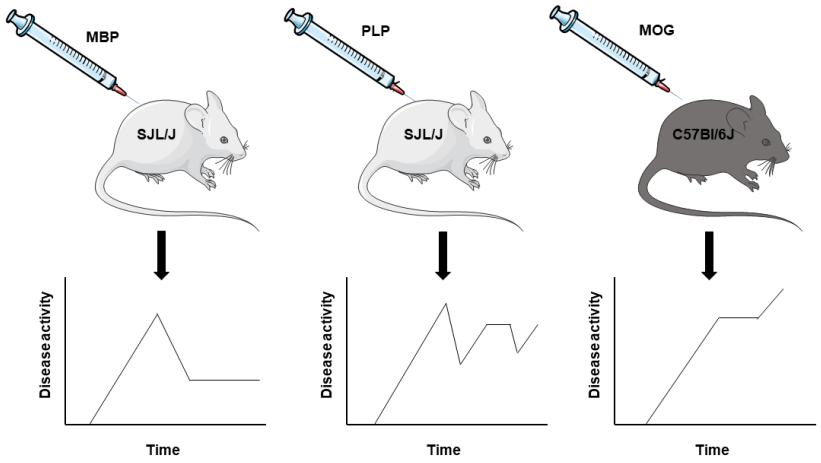

Figure 8: **Myelin proteins induce different clinical types of experimental autoimmune encephalomyelitis.** Myelin basic protein (MBP) induces an acute paralytic disease phenotype followed by complete or partial recovery in SJL/J and PL/J mice [329]. Proteolipid protein (PLP) induces a disease phenotype characterized by acute paralysis followed by remission and new attacks in SJL/J mice [329]. Myelin oligodendrocyte glycoprotein (MOG) induces a chronic progressive disease also characterized by paralysis in multiple mouse strains including C57Bl/6J [329]. Illustration elements was obtained from Servier Medical ART with license; https://creativecommons.org/licenses/by/3.0/. No changes were made to the elements.

In *manuscript III*, *V* eight-week old female mice and in *manuscript IV* rats were used because MS affects young women more frequently than men [194]. In *manuscript III* and *V*, we immunized C57Bl/6J mice with MOG-peptide to induce a chronic progressive disease phenotype [210,279] as no treatment is available for SPMS and only one is approved for the treatment of PPMS (**Table 7**). In *manuscript IV*, Lewis rats were immunized with MBP [204], which induced a highly aggressive, progressive disease phenotype [203]. Mice were weighted daily and evaluated for clinical symptoms of EAE by a blinded experimenter and given a score between 0 to 5 as previously described [204]. The clinical-relevant behavioural tests described in section 3.2 and 3.3 would have been relevant to apply in the EAE-studies. However, the clinical tests were not established at the time of the conduction of the EAE studies. The tests that were applied in the different experimental setups are summarized in **Table 10**. All the clinical-relevant behavioural tests have advantages and disadvantages such as time to conduct, required time between repeated measures, and price of experimental equipment, objectivity of measures and translational value.

Table 10: Clinical-relevant behavioural tests and measures used in the SOD1 G93A, rotenone, *Park2* and EAE experiments.

Tests used	Component evaluated	SOD1 experiments	Rotenone and *Park2* experiments	EAE experiments
Clinical score	Neurological function	x		x
Weight	Malaise	x	x	x
Hangwire test	Muscle strength, coordination	x		
Rotarod	Motor function, balance	x	x	
Grip strength	Muscle strength	x	x	
Cylinder test	Sensorimotor function	x	x	
Beam test + pole test	Sensorimotor function		x	
Y-maze test	Cognitive function	x	x	
Dark-light test	Anxiety		x	
NOR test	Cognitive function		x	

3.5. EVALUATION OF CLINICAL-RELEVANT BEHAVIOURAL TESTS

In *manuscript I, II* and *V* we have utilized multiple clinical behavioural tests as described in section 3.2 and 3.3. The rotarod and grip strength data was acquired in real time whereas the other tests described in **Table 8** and **Table 9** were recorded by a video camera and subsequently rated by three or four blinded assessors based on pre-established protocols.

Multiple key elements must be taken into account when performing biomedical experiments, and research in general, including validity ("*extent to which a measurement, test, or study measures what it purports to measure*" [332]), reliability (consistency of test results [333]), accuracy ("*the closeness of the measured value and true value*" [334]) and precision ("*refers to the consistency of repeated results*" [335]).

The clinical-relevant behavioural tests evaluate surrogates for different behavioural functions such as latency to fall of the rotarod as a measure of motor function or spontaneous alternation percentage in the Y-maze as a surrogate for cognitive function. However, all the described clinical-relevant tests have been validated, at least to some degree, as described in the references in **Table 8** and **Table 9**.

In order to assess the reliability of the clinical-relevant behavioural tests the reproducibility of the results from the clinical tests were evaluated based on data from two separate experiments using healthy male C57Bl/6J mice (**Figure 9**). The data illustrates that the results from the clinical-relevant behavioural tests was reproducible and that no significant differences between the repeated experiments were present except for the number of entries in the Y-maze. However, a Pearson's correlation analyses showed no significant correlation between number of entries and the surrogate measure of cognitive function (spontaneous alternation percentage, SAP). This is critical, as this illustrates that the difference in number of entries between the two experiments do not influence the outcome measure, SAP.

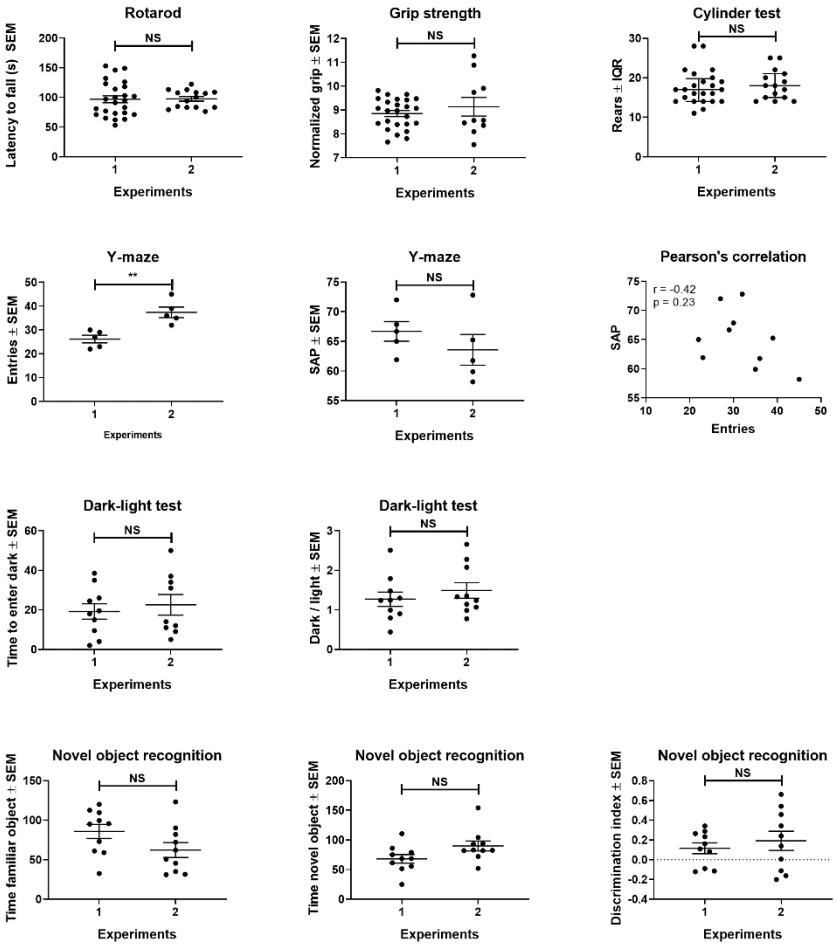

Figure 9: **Reproducibility of results from clinical-relevant behavioural tests described in Table and Table 9.** Scatterplot illustrating measure from independent animals in two different experiments. NS=no significant difference between groups. SEM=Standard error of mean. IQR=Interquartile range of median. SAP=Spontaneous alternation percentage. Normality and equality of variance was evaluated by Shapiro-Wilkinson test and *F*-test, respectively. Unpaired two-tailed t-test was used to assess significant differences if data was normal distributed and if not, Mann-Whitney U-test was applied. ** p = < 0.01.

Following the evaluation of reproducibility, precision of rotarod and grip strength measures (intra-test variation) was examined (**Figure 10a-b**). The grip strength test was repeated five times for each mouse in the same test session, which resulted in a mean intratest coefficient of variation (CV) of 9.7% (**Figure 10a**). This is an acceptable CV based on the treat-NMD standard operating procedure for using grip strength meter [336]. The rotarod test was repeated three times for each mouse in the

same test session giving an intratest CV for the mean latency to fall of 17.7% (**Figure 10b**). This is in accordance with previously published CV analyses showing a mean rotarod CV of 20.7% for male C57Bl/6J mice [337].

In addition, to evaluate the reliability of the pre-established protocols for analyses of video-based clinical-relevant behavioural tests, eight volunteer medical students rated videos from clinical behavioural tests from 10 male C57Bl/6J mice to assess the interrater variation. To assess interrater variation for the cylinder test, y-maze, dark-light box test and novel object recognition test, a CV was calculated for each test (**Figure 10c-l**). The cylinder test showed a mean interrater CV of 11.5% (**Figure 10c**). This is acceptable based on previously findings indicating a CV of up to 47.1% of rearing in open fields [337]. The y-maze mean interrater CV for number of entries and spontaneous alternation percentage was 12.1% and 8.1% respectively (**Figure 10d-e**). A Pearson correlation analyses found no correlation between number of entries and spontaneous alternation percentage, showing no association between number of entries and visuospatial memory (**Figure 10f**). This is crucial, as we want to evaluate visuospatial memory independent of entries. The y-maze mean interrater CV has previously been estimated to 14.9% in male mice [337]. The dark-light box test revealed a mean interrater CV of 6.6%, 3.9% and 6.8% for number of transitions, time spent in dark, and time spent in light respectively (**Figure 10g-i**). The novel object recognition test showed a mean interrater CV of 17%, 13.3% and 7.6% for time at familiar object, novel object and time spent at novel compared to total time exploring (**Figure 10j-l**).

The accuracy of the different clinical behavioural tests are difficult to estimate as the measures differ from one laboratory to another and as no golden-standard or reference value exists for e.g. the latency to fall of the rotarod or number of rears in the cylinder test for C57Bl/6J male mice. However, based on the above description it can be concluded that the pre-established protocols result in reliable results with acceptable variations.

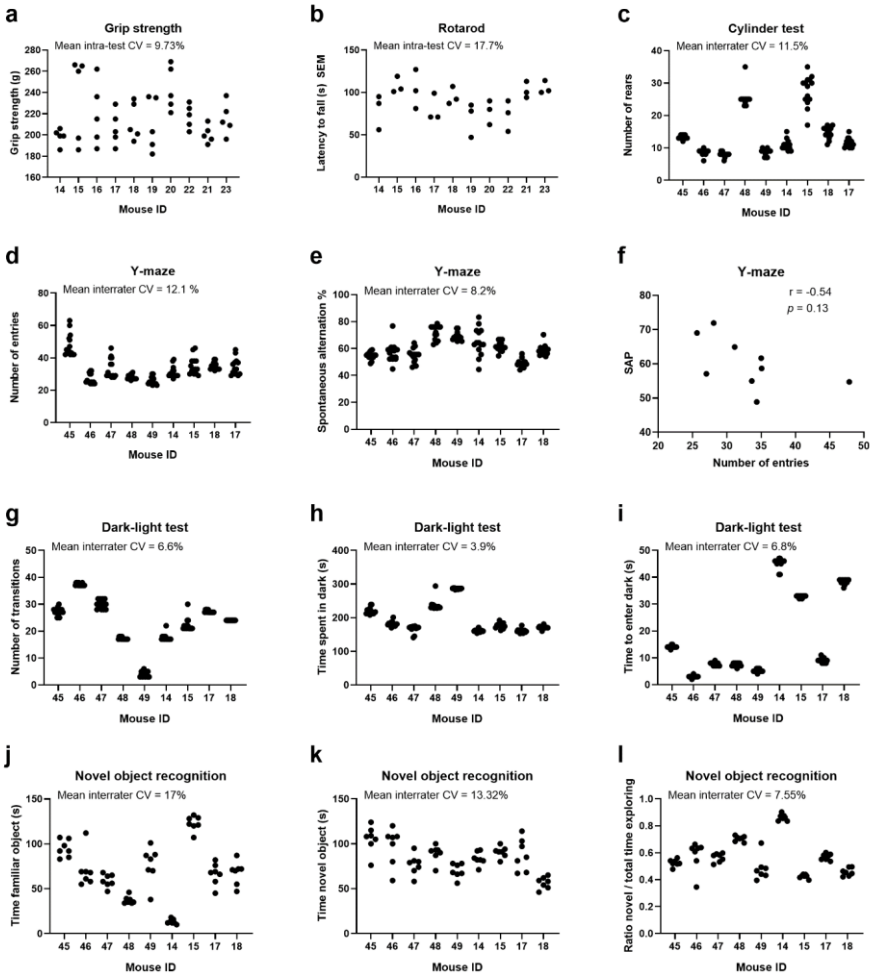

Figure 10: **Analyses of intratest and interrater variation in clinical-relevant behaviour tests used in SOD1 G93A, rotenone and *Park2* mouse models based on 10 male C57Bl/6J mice**. **a)** Scatterplot illustrating five repeated grip strength measures and mean coefficient of variation (CV). **b)** Scatterplot illustrating three repeated rotarod measures and mean CV. **c)** Scatterplot illustrating interrater variation in number of rears in the cylinder test and mean interrater CV. **d)** Scatterplot illustrating interrater variation in number of entries in the y-maze test and mean interrater CV. **e)** Scatterplot illustrating interrater variation in spontaneous alternation percentage (SAP) in the y-maze test and mean interrater CV. **f)** Pearson correlation test illustrating no correlation between number of entries and SAP. **g)** Scatterplot illustrating interrater variation in number of transitions in the dark-light test and mean interrater CV. **h)** Scatterplot illustrating interrater variation in time spent in dark in the dark-light test and mean interrater CV. **i)** Scatterplot illustrating interrater variation in time to enter dark in the dark-light test and mean interrater CV. **j)** Scatterplot illustrating interrater variation in time spent at the

familiar object in the novel object recognition test and mean interrater CV. **k)** Scatterplot illustrating interrater variation in time spent at the novel object in the novel object recognition test and mean interrater CV. **l)** Scatterplot illustrating interrater variation in ratio time spent at novel object compared to total time exploring in the novel object recognition test and mean interrater CV.

CHAPTER 4. RESULTS

This chapter includes the abstracts from *manuscript I – V*. The results are presented in the individual manuscripts in appendix A – E.

4.1. MANUSCRIPT I

Downregulating carnitine palmitoyl transferase 1 affects disease progression in the SOD1 G93A mouse model of ALS

Michael Sloth Trabjerg[1], Dennis Christian Andersen[1], Pam Huntjens[1], Kirsten Egelund Oklinski[1], Luise Bolther[1], Jonas Laugård Hald[1], Amalie Elton Baisgaard[1], Kasper Mørk[1], Nikolaj Warming[1], Ulla Bismark Kullab[1], Lona John Kroese[2], Colin Eliot Jason Pritchard[2], Ivo Johan Huijbers[2], John Dirk Vestergaard Nieland[1]

[1] *Department of Health Science and Technology, Aalborg University, 9220 Aalborg, Denmark*

[2] *Mouse Clinic for Cancer and Aging Research, Transgenic Facility, The Netherlands Cancer Institute, Amsterdam, 1066, Netherlands*

Manuscript submitted.

Abstract:

Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease characterized by death of motor neurons. The etiology and pathogenesis remains elusive despite decades of intensive research. Herein, we report that dysregulated metabolism plays a central role in the SOD1 G93A mouse model mimicking ALS. Specifically, we report that the activity of carnitine palmitoyl transferase 1 (CPT1) lipid metabolism is associated with disease progression. Downregulation of CPT1 activity by pharmacological and genetic methods results in amelioration of disease symptoms, inflammation, oxidative stress and mitochondrial function, whereas upregulation by high-fat diet or corticosterone results in a more aggressive disease progression. Finally, we show that downregulating CPT1 shifts the gut microbiota communities towards a protective phenotype in SOD1 G93A mice. These findings reveal that metabolism, and specifically CPT1 lipid metabolism plays a central role in the SOD1 G93A mouse model and shows that CPT1 might be a therapeutic target in ALS.

4.2. MANUSCRIPT II

Downregulation of carnitine palmitoyl-transferase 1 is highly efficacious in mouse models mimicking Parkinson's disease

Michael Sloth Trabjerg[1], Dennis Christian Andersen[1], Pam Huntjens, Kasper Mørk[1], Nikolaj Warming[1], Ulla Bismark Kullab[1], Marie-Louise Nibelius Skjønnemand[1], Michal Krystian Oklinski[1], Kirsten Egelund Oklinski[1], Luise Bolther[1], Lona J. Kroese[2], Colin E.J. Pritchard[2], Ivo J. Huijbers[2], Angelique Corthals[3], John Dirk Vestergaard Nieland[1]

[1] *Laboratory of Metabolism Modifying Medicine, Department of Health Science and Technology, Aalborg University, Denmark*

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[3] *Department of Science, John Jay College of Criminal Justice, City University of New York, New York, NY 10019, USA*

Manuscript submitted.

Abstract:

Glucose metabolism is dysregulated in Parkinson's disease (PD) causing a shift towards the metabolism of lipids. Carnitine palmitoyl transferase 1A (CPT1A) is the regulating step in metabolism of long chain fatty acids.. The aim of this study was to evaluate whether mice with a *Cpt1a* P479L mutation are resistant to chronic rotenone exposure. We further investigated the effects of downregulating CPT1 in chronic rotenone mouse models using C57Bl/6J, and *Park2* knockout mice. Here, we show that *Cpt1a* P479L mutant mice are resistant to rotenone-induced PD, and that inhibition of CPT1 is able of restoring neurological function, normal glucose metabolism, and alleviate markers of PD in the midbrain. Furthermore, we show that downregulation of the metabolism of lipids via CPT1 alleviates pathological motor and non-motor behavior, oxidative stress, and disrupted glucose homeostasis in *Park2* knockout mice. Finally, we confirm that rotenone induces gut dysbiosis in C57Bl/6J, and for the first time, in *Park2* knockout mice. We show that this dysbiosis is alleviated by the downregulation of the lipid metabolism via CPT1.

4.3. MANUSCRIPT III

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

Anne Skøttrup Mørkholt[1], Michael Sloth Trabjerg[1], Michal Krystian Egelund Oklinski[1], Luise Bolther[1], Lona John Kroese[2], Colin Eliot Jason Pritchard[2], Ivo Johan Huijbers[2], John Dirk Vestergaard Nieland[1]

[1] *Laboratory of Metabolism Modifying Medicine, Department of Health Science and Technology, Aalborg University, Denmark*

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Manuscript published in *Scientific Reports* volume 9, Article number: 13299 (2019).

Abstract:

Human mutations in carnitine palmitoyl transferase 1A (*CPT1A*) are correlated with a remarkably low prevalence of multiple sclerosis (MS) in Inuits *(P479L)* and Hutterites *(G710E)*. To elucidate the role of CPT1A, we established a *Cpt1a P479L* mouse strain and evaluated its sensitivity to experimental autoimmune encephalomyelitis (EAE) induction. Since CPT1a is a key molecule in lipid metabolism, we compared the effects of a high-fat diet (HFD) and normal diet (ND) on disease progression. The disease severity increased significantly in WT mice compared to that in *Cpt1 P479L* mice. In addition, WT mice receiving HFD showed markedly exacerbated disease course when compared either with *Cpt1a P479L* mice receiving HFD or WT control group receiving ND. Induction of EAE caused a significant decrease of myelin basic protein expression in the hindbrain of disease affected WT mice in comparison to *Cpt1a P479L* mice. Further, WT mice showed increased expression of oxidative stress markers like *Nox2* and *Ho-1*, whereas expression of mitochondrial antioxidants regulator *Pgc1α* was increased in *Cpt1a P479L* mice. Our results suggest that, lipids metabolism play an important role in EAE, as shown by the higher severity of disease progression in both WT EAE and WT EAF HFD-fed mice in contrast to their counterpart *Cpt1a P479L* mutant mice. Interestingly, mice with downregulated lipid metabolism due to the *Cpt1a P479L* mutation showed resistance to EAE induction. These findings support a key role for CPT1A in the development of EAE and could be a promising target in MS treatment.

4.4. MANUSCRIPT IV

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

Anne Skøttrup Mørkholt[1], Kenneth Kastaniegaard[1], Michael Sloth Trabjerg[1], Gopana Gopalasingam[1], Wanda Niganze[1], Agnete Larsen[2], Allan Stensballe[1], Søren Nielsen[1], John Dirk Nieland[1]

[1] *Department of Health Science and Technology, Aalborg University, Aalborg, Denmark*

[2] *Department of Biomedicine, Aarhus University, Aarhus, Denmark*

Manuscript published in *Scientific Reports* volume 8, Article number: 7092 (2018).

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 aetiology 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.

4.5. MANUSCRIPT V

Dysregulation of metabolic pathways by carnitine palmitoyl-transferase 1 plays a key role in central nervous system disorders: experimental evidence based on animal models

Michael Sloth Trabjerg[1], Anne Skøttrup Mørkholt[1], Jacek Lichota[1], Michal Krystian Egelund Oklinski[1], Dennis Christian Andersen[1], Katrine Jønsson[3], Kasper Mørk[1], Marie-Louise Nibelius Skjønnemand[1], Lona John Kroese[2], Colin Eliot Jason Pritchard[2], Ivo Johan Huijbers[2], Parisa Gazerani[1], Angelique Corthals[4], John Dirk Vestergaard Nieland[1]

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Manuscript published in *Scientific Reports* volume 10, Article number: 15583 (2020).

Abstract:

The etiology of CNS diseases including multiple sclerosis, Parkinson's disease and amyotrophic lateral sclerosis remains elusive despite decades of research resulting in treatments with only symptomatic effects. In this study, we provide evidence that a metabolic shift from glucose to lipid is a key mechanism in neurodegeneration. We show that, by downregulating the metabolism of lipids through the key molecule carnitine palmitoyl transferase 1 (CPT1), it is possible to reverse or slowdown disease progression in experimental models of autoimmune encephalomyelitis-, $SOD1^{G93A}$ and rotenone models, mimicking these CNS diseases in humans. The effect was seen both when applying a CPT1 blocker or by using a *Cpt1a P479L* mutant mouse strain. Furthermore, we show that diet, epigenetics, and microbiota are key elements in this metabolic shift. Finally, we present a systemic model for understanding the complex etiology of neurodegeneration and how different regulatory systems are interconnected through a central metabolic pathway that becomes deregulated under specific conditions.

CHAPTER 5. DISCUSSION

Neurodegenerative diseases such as ALS, PD and MS might seem as completely unrelated diseases at first glance, however they share common pathogenic mechanisms including, but not limited to, oxidative stress, mitochondrial dysfunction, neuroinflammation, dysregulated HPA-axis and gut dysbiosis (**Table 2, 4, 6**). Additionally, these and other neurodegenerative diseases are characterized by decreased glucose metabolism and upregulated lipid metabolism. The results in the individual manuscripts are discussed in the individual manuscripts. Therefore, in this chapter the main findings will be discussed based on a systemic platform showing how dysregulated metabolism can explain the activation and exacerbation of the aforementioned disease mechanisms.

5.1. SYSTEMIC PLATFORM FOR NEURODEGENERATIVE DISEASES

CNS diseases are classified according to their clinical or neuropathological characteristics such as motor neuron disease, demyelinating disease or movement disorder. Multiple treatments exist for ALS (**Table 3**), PD (**Table 5**) and MS (**Table 7**) targeting specific mechanisms of these diseases, but despite significant effort, none of the available treatments can stop the progression of the specific disease, let alone curing them. Thus, targeting one mechanism is not enough to halt the progression of these diseases. In recognition of this characteristic, we propose a multisystem framework [279], which hypothesizes how CNS and peripheral disease mechanisms are linked to metabolic dysregulation and how these mechanisms are interconnected (**Figure 11**). Some of the mechanisms have been presented previously [26] by my colleague and collaborator *Anne Skøttrup Mørkholt* but in this thesis, this model is expanded considerably. Using this model, or platform, can facilitate experiments to further elucidate the aetiology of neurodegenerative diseases, and thereby possibly identifying novel pharmaceutical targets. Metabolic dysregulation in neurodegenerative diseases was reported several decades ago but this has, as we believe with regret, not been used as a major focus for the understanding or treatment of neurodegenerative diseases.

Glucose and lipid metabolism, under normal conditions are in balance. However, this balance can be shifted from a more glucose metabolism to a predominant lipid metabolism by a variety of physiological, psychological or pathological mechanisms/stressors for example infections [338,339], diet [340], chronic psychological stress and hypoxia [341] based on the individual's genetics. The metabolic shift is partly regulated by PPARs [8]. This shift results in a cascade of reactions, which initiates a vicious cycle where all the pathological processes are directly or indirectly linked.

Therefore, the systemic platform consists of a complicated network with multiple interconnections.

In the illustration (**Figure 11**), pathological mechanisms are highlighted in gray circles. Here each one is explained as how it can contribute to a shift in metabolism and thereby contribute to the development of neurodegeneration.

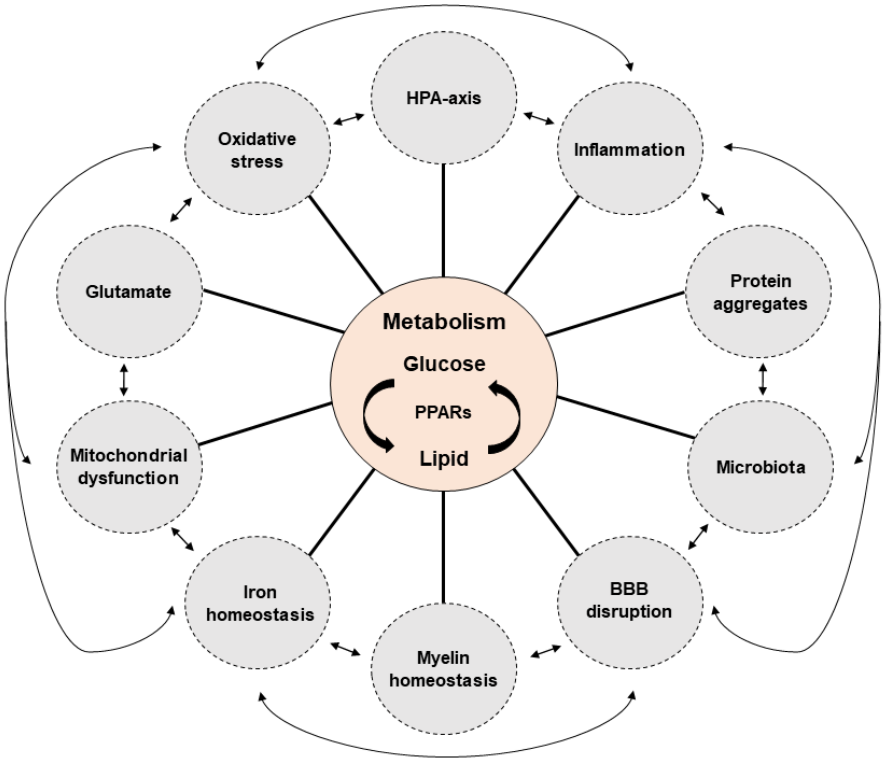

Figure 11: **Schematic illustration of the multisystem framework for the establishment and progression of neurodegenerative diseases**. One or multiple environmental stressors result in a shift towards lipid metabolism, which initiates a cascade reaction resulting in disruption of homeostasis and establishment of several pathogenic pathways e.g. inflammation, oxidative stress and mitochondrial dysfunction. Inspired by and extended based on [279].

5.1.1. INFLAMMATION AND HPA-AXIS DISRUPTION

The metabolic shift results in inflammation due to activation of dendritic cells, macrophages, T helper 1- and T helper 17-cells [265,342]. The immune cells produce an acute inflammatory response, which increases mobilization and metabolism of lipids due to secretion of inflammatory cytokines [343]. Additionally, increased lipid

metabolism results in polarization of microglia and macrophages towards a phagocytic phenotype [344], which can have detrimental effects on ageing processes [345].

The immune system can directly activate the HPA-axis inside the CNS but also peripheral inflammation can activate the axis by a variety of mechanisms and molecules including myeloid differentiation primary response 88, cyclooxygenase-2 and prostaglandin E2 [35]. The activation of the HPA-axis due to an acute stress response results in secretion of glucocorticoids, catecholamines and mineralocorticoids, which have short-term beneficial effects promoting survival [35]. However, a chronic activation of the HPA-axis and production of cortisol can result in pro-inflammatory mechanism inside the CNS and in the periphery [35], possibly due to failure of the negative feedback mechanisms [37]. Glucocorticoids also induce oxidative stress in neurons by increasing oxidation, mitochondrial membrane potential, calcium levels and NOX expression and simultaneously downregulate anti-oxidative defense mechanisms [346], which result in neuronal death. The induction of oxidative stress results in an exacerbation of the HPA-axis activity due to failure of negative feedback mechanisms [346]. In addition, high levels of glucocorticoids result in insulin resistance and lipolysis [36,346], which further aggravates the vicious cycle. Accordingly, cortisol increases lipolysis and β-oxidation in humans [347]. Prenatal stress also disrupts the HPA-axis and induce profound changes in the gut microbiome *in vivo* [348,349].

5.1.2. OXIDATIVE STRESS

Hypoxia decreases the activity of pyruvate dehydrogenase complex through the upregulation of pyruvate dehydrogenase kinase 1 by hypoxia-inducible-factor 1 [350], which could induce a shift towards lipid metabolism [341]. Additionally, the metabolic shift towards lipid metabolism induces increased production of ROS directly but also indirectly as lipid metabolism requires more oxygen [15]. Oxidative stress leads to mitochondrial dysfunction characterized by loss of the membrane potential in the mitochondria and disrupted ion gradients [15]. This accumulation results in termination of ATP production and thus cell death [28]. Chronic intermittent hypoxia induce oxidative stress and upregulate pro-inflammatory cytokines such as IL-6 and TNF-α in the CNS [351]. Accordingly, obstructive sleep apnea in humans results in chronic intermittent hypoxia inducing increased level of oxidative stress serum markers [352]. In addition, increased glucocorticoids can result in oxidative stress as described above [346]. Activated microglia and chronic inflammation can result in the production of ROS and RNS, further exacerbating oxidative stress [353]. However, oxidative stress can also activate the immune system and induce an inflammatory response [354].

5.1.3. GLUTAMATE

Glutamate is a primary excitatory neurotransmitter in the CNS and high levels of glutamate result in excitotoxicity causing cell death [29,67]. Oxidative stress (e.g. exposure to 4-hydroxy-2-nonenal) can inhibit the extracellular reuptake of glutamate [355]. In addition, glutamate is able to induce oxidative stress and neurodegeneration in the CNS by lipid peroxidation [356]. Interestingly, glutamate is involved in the secretion of insulin and thereby glucose homeostasis [357] and hypoxia induces synthesis of fatty acids from glutamate in the CNS [358]. High levels of glutamate can induce mitochondrial dysfunction due to overstimulation of the N-methyl-D-aspartate receptors resulting in Ca^{2+} overload [359]. Additionally, inflammation is hypothesized to result in glutamate "spillover" from glia cells [360], which could result in exacerbation of excitotoxicity, oxidative stress, mitochondrial dysfunction and thereby neurodegeneration.

5.1.4. MITOCHONDRIAL DYSFUNCTION

Increased lipid metabolism *in vivo* due to a diet with a high content of saturated fat and glucose induces disruption of mitochondrial biogenesis by downregulation of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), lipid peroxidation, decreased mitochondrial DNA, hyperglycemia, insulin resistance, decreased PPARα expression and neuropathic symptoms [361]. In addition, HFD exacerbate disease activity and progression in multiple *in vivo* models of neurodegenerative diseases [210,362–364]. Additionally, mitochondrial dysfunction is linked to oxidative stress and inflammation as described above.

5.1.5. IRON

Iron is essential for a variety of process involved in homeostasis including oxygen transport, regulation of cell growth, transport of electrons, DNA synthesis and metabolism [365]. Iron has been associated with insulin resistance and metabolic syndrome in multiple meta-analysis [365]. In this regard, increased ferritin levels has been associated with insulin resistance *in vivo*, in humans and *in vitro* [366]. Further, increased iron levels are associated with increased triglycerides, glucose, decreased HDL, increased levels of pro-inflammatory cytokines including IL-6, TNF-α, IL-1β, lipid peroxidation and mitochondrial dysfunction in the liver *in vivo* [367]. Iron is crucial for the metabolism in the myelin-producing oligodendrocytes but increased iron accumulates in the major myelinated tracts in the CNS and results in downregulation of myelin-associated genes causing demyelination and neurodegeneration [368,369].

5.1.6. MYELIN

The synthesis of myelin requires vast amount of lipids including cholesterol, which is present at the myelin membrane in high quantities [41,369]. Thus increased lipid

metabolism can result in delipidation of the myelin proteins that can activate the immune system due to autoimmune mechanisms causing inflammation and demyelination [33,204]. In addition, lipids are targets for oxidative stress causing lipid peroxidation [28].

5.1.7. BBB

The BBB is crucial for the protection of the CNS from toxins, pathogens and to maintain homeostasis in the CNS by tightly regulating the passage of e.g. nutrients and ions [370]. Therefore, disruption of the BBB has major pathological effects. High amounts of FAs can cause disruption of the BBB due to dysregulation of PPARs [8]. Accordingly, HFDs and obesity is associated with increases permeability of the BBB [371]. This results in entrance of immune cells and leakage of iron into the CNS, which results in inflammation and oxidative stress [369].

5.1.8. GUT MICROBIOTA

The gut microbiota has major functions in the maintenance of homeostasis including modulation of the lipid metabolism [279,372] . Disruption of the gut microbiota due to e.g. HFDs can result in local and systemic inflammation by increased levels of lipopolysaccharides and disruption of the intestinal barrier [373]. This can affect the brain directly due to disruption of the BBB and indirectly by the gut-brain-axis [42,373]. Metabolites produced by the gut microbiota can increase and decrease the permeability of the BBB [374]. Additionally, the HPA-axis influences the gut microbiota and *vice versa* [375]. In accordance, changes in the gut microbiota are associated with multiple neurodegenerative diseases including ALS, PD and MS [43,98,162] and amyloid-producing gut microbiota exacerbate α-syn pathology and disposition in the gut and CNS *in vivo* [376]. Further, fecal transplant from patients with PD into mice aggravates neuropathology and inflammation [162] . Thereby linking the gut microbiota to the other disease mechanisms.

5.1.9. PROTEIN AGGREGATION

Protein aggregates play a major role in neurodegenerative diseases as presented in chapter 1. Interestingly, decreased normal physiological α-syn levels are associated with insulin resistance and decreased glucose uptake *in vitro* and *in vivo* [377]. Low serum α-syn levels are associated with insulin resistance in humans [378]. In addition the fALS SOD1 G93A mutation are characterized by SOD1 aggregates and are also associated with metabolic dysregulation [80]. This indicates a possible relationship between metabolism and pathological protein aggregates [173,379]. In addition, the aggregates are associated with changes in the gut microbiome and inflammation [98,128,162].

In summary, based on the above description, it is evident that glucose-lipid metabolism plays a pivotal role in the establishment and progression of disease mechanisms involved in neurodegenerative diseases. Therefore, it seems reasonable to hypothesize that neurodegenerative disease are complex diseases involving multiple organ systems and pathological mechanisms, which all are associated to dysregulated metabolism. However, one key question is how do neurodegenerative diseases like ALS, PD and MS arise and based on the systemic platform, how many systems needs to be out of balance for a disease to develop?

It could be *speculated* that different combinations of mechanisms are responsible for the development of different neurodegenerative diseases but that the change in metabolism is a common initiator. However, based on speculations, not a disruption in a single system is enough to cause disease by itself, except for some gene mutations, and therefore a minimum of possibly three systems must be abrogated. Inflammation, diet and stress could for example be the pathological drivers in MS whereas it for example in PD could be mitochondrial dysfunction due to age, dysbiosis and stress. ALS could arise due to local hypoxia from a micro trauma in genetic susceptible people followed by inflammation, oxidative stress and mitochondrial dysfunction. In addition, multiple of these mechanisms are affected by epigenic factors, which potential also could account for some of the differences in these diseases. It's evident that ALS, PD and MS share common mechanism but the clinical presentation and pathological hallmarks are distinct but the mechanisms in the diseases can all be explained to some extent by the systemic platform.

Therefore, the next section will present findings from *manuscript I – V*, to present how different mechanism are involved in the different *in vivo* models mimicking ALS, PD and MS and that the tested hypotheses described in chapter 2 was, to some extent, approved correctly.

5.2. MANUSCRIPT I – V AND THE SYSTEMIC PLATFORM

Manuscript I – V deal with research underpinning the systemic platform proposed as a mechanistic description of the aetiology and pathology of neurodegenerative diseases. The detailed results are described in the manuscripts (Appendix A-E) [204,210,279]. Therefore, in this section the main findings from each manuscript will be summarized and discussed.

5.2.1. MANUSCRIPT I

Manuscript I (Appendix A)evaluates the role of dysregulated metabolism in the SOD1 G93A mouse model mimicking fALS. It presents an attempt to halt the disease progression in SOD1 G93A female mice by downregulation of CPT1 activity by applying the CPT1-blocker etomoxir (5mg/kg) from day 70. Etomoxir treated mice had significantly later disease onset, improved clinical scores, muscle strength,

sensory motor function and visuospatial memory compared to SOD1 G93A mice receiving placebo. However, no significant effect on survival was observed. This indicates that pharmacological inhibition of CPT1 slows down disease progression but that the SOD1 G93A mutation results in a too aggressive phenotype to be ameliorated by etomoxir. Accordingly, Ranolazine, another CPT1 antagonist, was recently shown to increase muscle strength and to slow down disease progression by amelioration of muscle hypermetabolism in SOD1 G93A mice [380]. Etomoxir was able to counteract increased serum levels of glucose and re-establish normal lipoprotein balance. SOD1 mice receiving placebo or etomoxir both had decreased levels of Choline O-acetyltransferase (ChAT) in the spinal cord, indicating death of MNs, as expected based on the clinical data. However, etomoxir diminished the levels of the pro-inflammatory cytokines IL-1β and TNF-α and decreased the gene expression of inflammatory markers in the spinal cord. In addition, based on gene expression, etomoxir restored mitochondrial biogenesis, increased glucose metabolism, myelination and oxidative stress defence. Further, etomoxir decreased denervation, inflammation and increased oxidative stress defence in tibialis anterior muscle tissue. Accordingly, the downregulation of β-oxidation, has been shown to decrease the level of inflammatory cytokines and increase oxidative stress defence *in vitro* [381]. In addition, immunohistochemistry was performed to evaluate changes in CPT1A, CPT1C, MBP, GFAP, IBA1 and ChAT labelling, which was consistent with the ELISA and gene expression data.

To dissect the mechanism and role of CPT1A, we crossed SOD1 G93A mice with *Cpt1a* P479L mice to obtain SOD1 G93A mice with heterozygote (SOD1 $G93A^{Wt/Cpt1a}$) and homozygote (SOD1 $G93A^{Cpt1a/Cpt1a}$) *Cpt1a* P479L mutations. The SOD1 $G93A^{Wt/Cpt1a}$ and SOD1 $G93A^{Cpt1a/Cpt1a}$ mice had significant later onset of tremor, lower clinical score, increased muscle strength, sensorimotor function and visuospatial memory compared to SOD1 G93A mice. Following day 130, the clinical benefits of the decreased CPT1A activity diminished and the symptoms progressed regardless of genotype. However, we observed increased survival in the SOD1 $G93A^{Wt/Cpt1a}$ and SOD1 $G93A^{Cpt1a/Cpt1a}$ mice. Downregulation of CPT1A activity in SOD1 G93A mice by crossing in the *Cpt1a* P479L mutation resulted in amelioration of hyperglycaemia and restored lipoprotein levels in serum. In addition, the level of ChAT was upregulated in the spinal cord of SOD1 $G93A^{Cpt1a/Cpt1a}$ mice. SOD1 $G93A^{Wt/Cpt1a}$ and SOD1 $G93A^{Cpt1a/Cpt1a}$ mice had lower levels of the reactive microglia/macrophage marker C3XCR1, increased levels of the anti-inflammatory cytokine IL-10 and decreased levels of pro-inflammatory cytokines based on ELISA experiments. Accordingly, based on gene expression, mitochondrial biogenesis, glucose metabolism, myelination and oxidative stress defence mechanisms was upregulated in the SOD1 G93A mice with genetic downregulated CPT1A activity. Additionally, muscle atrophy and markers of denervation was decreased in SOD1 $G93A^{Wt/Cpt1a}$ and SOD1 $G93A^{Cpt1a/Cpt1a}$ mice. This was consistent with increased levels of the anti-inflammatory cytokine IL-10 and decreased levels of IL-1β and TNF-α. Based on this, we propose that CPT1A activity plays a role in the modulation of

inflammation, metabolism and oxidative stress in the SOD1 G93A mouse model. In addition, we propose, based on the etomoxir and *Cpt1a* P479L x SOD1 G93A experiments, that CPT1 plays a central role in the SOD1 G93A mouse model at least in early and mid-stage disease.

In conjunction with the clinical-relevant effects of downregulating CPT1 and CPT1A activity in SOD1 G93A mice, we examined the effects of stimulating lipid metabolism and promoting insulin resistance by feeding SOD1 G93A mice a 60% HFD versus a normal diet (ND). The HFD resulted in significant earlier onset of tremor, decreased muscle strength, sensorimotor function and loss of visuospatial memory compared to SOD1 G93A mice receiving ND. Additionally, the SOD G93A mice receiving HFD had a significantly decreased survival compared to the ND group. HFD resulted in increased serum glucose levels and disrupted lipoprotein homeostasis characterized by increased LDL and LDL/HDL ratio. Further, the level of ChAT and IL-10 was decreased whereas IL-1β was increased in the spinal cord. In addition, gene expression indicated decreased mitochondrial biogenesis, inflammation and oxidative stress in the spinal cord in the SOD1+HFD mice. SOD1+HFD mice were also characterized by increased muscle atrophy, denervation and inflammation and decreased glucose metabolism in the tibialis anterior muscle. Thus, the data indicate that HFD results in a more severe disease progression, which is in accordance with previous studies in *in vivo* models of neurodegenerative diseases [362–364].

As described in chapter 1, dysregulation of the HPA-axis has been recognized to play a role in ALS and therefore the effect of administering 20 mg/kg CORT in SOD1 G93A mice was evaluated from day 70 and until day 100. CORT resulted in significant earlier onset of tremor, increased clinical scores, decreased muscle strength, sensorimotor function and visuospatial memory compared to SOD1 G93A not receiving CORT. CORT resulted in increased glucose levels in serum and increased gene expression of inflammatory and oxidative stress markers in the spinal cord. In addition, CORT resulted in decreased expression of glucose metabolism in tibialis anterior. Therefore, it seems likely that increased levels of glucocorticoids results in a more severe disease progression in the SOD1 G93A fALS model. This is in agreement with Fidler et al. (2011), who demonstrated that increased levels of CORT due to chronic restraint stress resulted in exacerbation of disease and inflammation in the SOD1 G93A mouse model [90].

Finally, *manuscript I* evaluates changes in the gut microbiome in SOD1 G93A. SOD1 G93A mice have alternations in their gut microbiome from early stages of the disease [98], which is attenuated by etomoxir and homozygote *Cpt1a* P479L mutation and aggravated by HFD and CORT. This illustrates that the gut microbiome may play a role in the establishment and progression of disease in the SOD1 G93A mouse model. The overall effects of downregulating and upregulating CPT1 activity in the SOD1 G93A mouse model are summarized in **Figure 12**. However, some limitations apply to this study such as the fact that it is at the moment unknown in, which cell types

(glial or neuronal) the CPT1 target engagement results in the amelioration of some of the disease mechanisms including inflammation and oxidative stress.

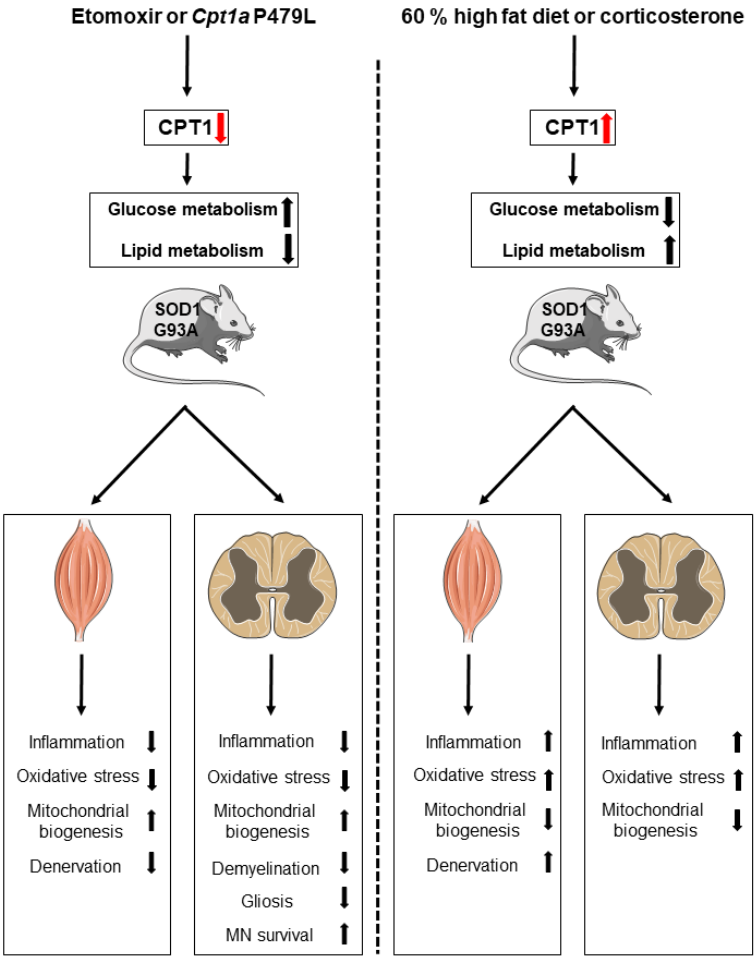

Figure 12: The effects of downregulating and upregulating CPT1 activity in the SOD1 G93A mouse model. Downregulation of CPT1 activity resulted in amelioration of clinical-relevant symptoms and e.g. diminished inflammation, oxidative stress and increased mitochondrial biogenesis. Upregulation of CPT1 activity resulted in exacerbation of clinical-relevant symptoms and e.g. increased inflammation, oxidative stress and decreased mitochondrial biogenesis. Illustration elements was obtained from Servier Medical ART with license; https://creativecommons.org/licenses/by/3.0/. No changes were made to the elements.

5.2.2. MANUSCRIPT II

Manuscript II (Appendix B)evaluates the role of dysregulated metabolism in rotenone and *Park2* models mimicking environmental-induced and early-onset familial PD. It covers an attempt to halt the disease progression in the models by downregulation of CPT1 activity by using the *Cpt1a* P479L mutated mice or by etomoxir (5mg/kg). Further, the manuscript combines the *Park2* mouse model with a chronic rotenone regimen to mimic gene-environment interactions.

First, the manuscript presents that *Cpt1a* P479L mice are resistant to the chronic rotenone model based on rotarod, grip strength, cylinder test and spontaneous alternation in the y-maze. The resistance was associated with increased levels of TH and dopamine in the midbrain compared to wild type mice receiving rotenone. Additionally, the *Cpt1a* P479L mice receiving rotenone did not develop hyperglycaemia and alternations in LDL and LDL/HDL ratio. Further, *Cpt1a* P479L mice exposed to rotenone had decreased gene expression of reactive microglia/macrophage markers, possibly indicating lower inflammation.

Secondly, to underpin the role of CPT1, the manuscript investigates the effects of etomoxir in C57Bl/6J mice exposed to the chronic rotenone mouse model. Etomoxir treatment was initiated at day 32 and combined with an alternation between rotenone and treatment to investigate the effect during a continuous rotenone exposure. Etomoxir resulted in amelioration of motor symptoms, lowered LDL, increased HDL and decreased LDL/HDL ratio in serum. In addition, etomoxir treated mice had increased levels of TH in the striatum and midbrain and decreased α-syn in the midbrain. Further, to account for the fact that rotenone can induce temporary decreased TH levels, the manuscript evaluated the effect of etomoxir during a wash-out period following 32 days of rotenone administration. The etomoxir treated mice during the wash-out period had increased visuospatial memory compared to rotenone exposed mice receiving vehicle. However, we did not observe any significant difference in motor function. Further, etomoxir treated mice had decreased serum LDL and LDL/HDL ratio and decreased α-syn levels in the midbrain. In addition, the etomoxir treated mice had decreased gene expression of *Iba1* and *Cd68* in the midbrain, pointing towards decreased levels of reactive microglia/macrophages and thereby potential decreased inflammation. Thus, the *Cpt1a* P479L rotenone and rotenone-etomoxir experiments indicates that CPT1 plays a central role in the rotenone model.

Thirdly, *manuscript II* investigates the effect of downregulating CPT1 activity in the *Park2* mouse model, which is characterized by dysregulated glucose-lipid metabolism [324,325]. *Park2* mice received etomoxir (5mg/kg) for 21 days based on the etomoxir experiments in C57Bl/6J mice. Etomoxir increased muscle strength and attenuated cognitive and anxiety-like symptoms and decreased serum glucose levels compared to *Park2* mice receiving vehicle. No difference in TH, α-syn and dopamine levels in

the midbrain were observed. However, *Park2* mice are in general not characterized by pathological changes in TH and α-syn levels [323] but mitochondrial dysfunction [149], oxidative stress and inflammation [382]. In accordance, etomoxir treated *Park2* mice had lower gene expression of *Iba1* in the midbrain compared to mice receiving vehicle. In addition, the effect of downregulating CPT1 activity by etomoxir following a combined *Park2*-chronic rotenone exposure model was evaluated. *Park2* mice developed a more severe disease phenotype following rotenone exposure and etomoxir treatment only diminished recognition memory impairment, decreased serum LDL/HDL ratio and α-syn levels in the midbrain. However, Park2 mice exposed to rotenone and treated with etomoxir had decreased gene expression of *Iba1* and *Cd68*. Therefore, *manuscript II* also illustrates that CPT1 plays a role in the modulation of disease progression in the *Park2* mouse model mimicking early-onset PD but that the *Park2*-rotenone model might be too aggressive.

Finally, *manuscript II* investigates changes in the gut microbiome in the different rotenone and *Park2* mouse models. In both models, rotenone and *Park2* mutation induces changes in the gut microbiome, which is counteracted by downregulation of CPT1 by etomoxir or *Cpt1a* P479L mutation. This illustrates that the gut microbiome plays a role in the establishment and progression of disease in the chronic rotenone and *Park2* mouse models. The overall effects of downregulating the CPT1 activity in the chronic rotenone and *Park2* mouse model are summarized in **Figure 13**. However, some limitations apply to this study including, but not limited to, that the effects of downregulating CPT1 activity in the periphery was not investigated and that levels of inflammatory cytokines, mitochondrial biogenesis markers and oxidative stress proteins were not investigated in the midbrain.

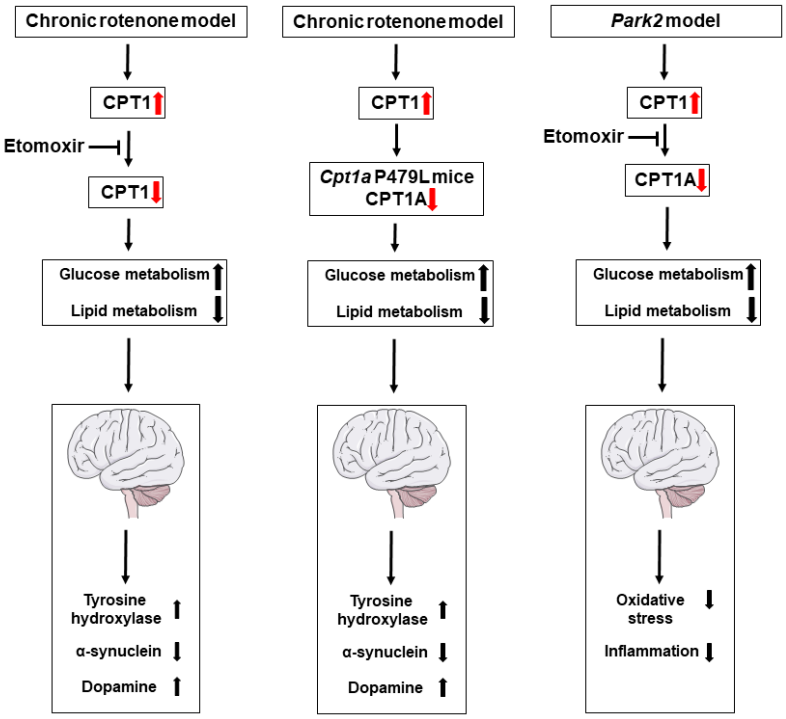

Figure 13: The effects of downregulating CPT1 activity in the chronic rotenone and *Park2* mouse model mimicking PD-like disease. Downregulation of CPT1 activity resulted in amelioration of clinical-relevant symptoms and e.g. increased tyrosine hydroxylase, dopamine and decreased α-synuclein, diminished inflammation and oxidative stress in the midbrain. Illustration elements was obtained from Servier Medical ART with license; https://creativecommons.org/licenses/by/3.0/. No changes were made to the elements.

5.2.3. MANUSCRIPT III

Section 1.6.3 presented that a large proportion of the Inuit's has a *CPT1A* P479L point mutation and a significant lower prevalence of MS, indicating a possible association between lipid metabolism and MS. To investigate if CPT1A activity plays a role in the development of MS a *Cpt1a* P479L mutant mouse strain was developed as previously described [210]. *Manuscript III* (Appendix C) evaluates whether *Cpt1a* P479L female mice show resistance against MOG-induced EAE and the role of 60% HFD in the MOG-induced EAE model.

Female *Cpt1a* P479L mice was resistant to MOG-induced EAE and presented with significant lower clinical score compared to wild type mice induced with EAE [210]. Following this, *manuscript III* tested the effect of 60% HFD and presented that HFD resulted in exacerbation of disease in wild type mice but not *Cpt1a* P479L mice based

on clinical scores [210]. Based on the different clinical effects of 60 % HFD on the EAE disease progression in wild type and *Cpt1a* P479L mice *manuscript III* investigated fluorescent MBP-staining in the brainstem and cerebellum. *Cpt1a* P479L mice had increased density of MBP in the brainstem and cerebellum. In addition, western blotting revealed increased MBP protein expression in the hindbrain of *Cpt1a* P479L mice compared to wild type EAE-induced mice. This indicates that downregulation of CPT1A activity results in protection from EAE-induced demyelination. Accordingly, Shriver et al. (2011) showed that etomoxir resulted in diminished demyelination *in vivo* [383]. Pathogenic mechanisms like oxidative stress and mitochondrial dysfunction are a hallmark of MS and EAE (as presented in section 1.5) and therefore *manuscript III* investigated changes in gene expression of oxidative stress (*Nox2*, *Ho-1* and *Nrf2*) and mitochondrial biogenesis (*Pgc1α*) markers. *Cpt1a* P479L mice induced with EAE had significant lower fold gene expression of the oxidative stress marker *Nox2* in the front-, mid- and hindbrain indicating lower oxidative stress due to the genetic downregulation of CPT1A [210]. Further, *Cpt1a* P479L mice induced with EAE had significant higher fold gene expression of *Pgc1α* in the hindbrain [210] indicating potential increased mitochondrial biogenesis in the *Cpt1a* P479L mutated mice. Therefore, *manuscript III* illustrates that CPT1A plays a central role in the disease induction and progression of EAE due to decreased demyelination and oxidative stress (**Figure 14**).

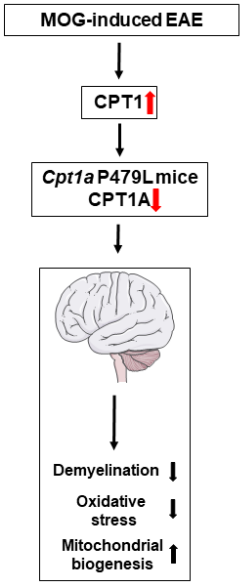

Figure 14: *Cpt1a* P479L mice are resistant to MOG-induced EAE. The downregulated CPT1A activity results in decreased demyelination, oxidative stress and increased mitochondrial biogenesis. Illustration elements was obtained from Servier Medical ART with license; https://creativecommons.org/licenses/by/3.0/. No changes were made to the elements.

5.2.4. MANUSCRIPT IV

The primary focus in MS pathogenesis has been autoreactive T-cells but B-cells and autoantibodies have been indicated to play substantial role in MS in recent years [198,241,258]. Therefore, *manuscript IV* (Appendix D) evaluates the autoantibody-antigen response in a MBP-induced rat EAE model and the effects of etomoxir and interferon-β on the autoantibody-antigen response.

First, the manuscript presents that serum from the EAE rats contain antibodies that recognize rat brain antigens and that the EAE rats have increased serum antibodies towards MBP [204]. This illustrates that autoantibodies are present in the EAE-induced rats mimicking MS and that the disease was successfully induced based on the autoantibodies towards MBP. Following the evaluation of the presence of autoantibodies, immunoprecipitation and label-free mass spectrometry was performed to investigate differences in autoantibody reactivity in the different treatment groups (placebo, etomoxir day 1 and 5 and interferon-β day 1 and 5). The placebo group had significant upregulated autoantibody reactivity towards multiple positive acute phase reactants and significant downregulation of autoantibody reactivity towards negative acute phase reactants compared to the control groups, which indicated increased inflammatory activity in the placebo rats [204] as expected based on published literature [203].

The etomoxir treated EAE rats had significantly lower antibody reactive towards multiple brain antigens such as apolipoprotein E (apoE) and serum amyloid P component [204]. ApoE knockout mice induced with EAE have an exacerbated disease course, increased mortality and infiltration of immune cells [384]. This could indicate that the etomoxir treated mice had increased levels of active apoE based on their clinical score [203,204]. Serum amyloid P component transgenic mice (overexpression) induced with EAE have ameliorated disease activity and decreased infiltration of immune cells in the CNS compared to non-transgenic controls and serum amyloid P component knockout mice have exacerbation of disease activity following EAE induction [385]. Therefore, the lower autoreactive towards serum amyloid P component could also explain the diminished disease activity in the etomoxir treated rats [203,204]. Overall, *manuscript IV* presents data confirming the relevance of autoantibodies in the MBP-induced rat EAE model and illustrates that etomoxir modulates the autoantibody response against CNS antigens (**Figure 15**). This underpins a connection between CPT1 lipid metabolism, inflammation and neurodegenerative diseases *in vivo*. However, one limitation to this study is that serum was used to investigate the autoantibody-reactivity towards brain antigens. It would be relevant also to assess the level of autoantibody-reactivity in the cerebrospinal fluid of the rats.

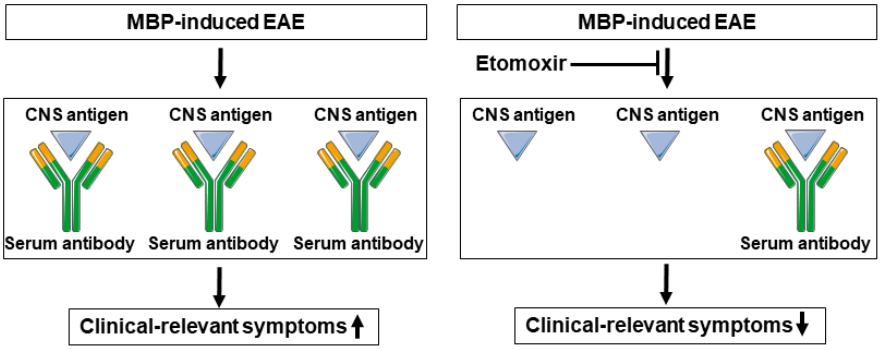

Figure 15: MPB-induced EAE results in generation of autoantibodies recognizing brain-antigens. The autoantibody-brain-antigen recognition is modulated by Etomoxir and associated with decreased clinical-relevant disease symptoms. Illustration elements was obtained from Servier Medical ART with license; https://creativecommons.org/licenses/by/3.0/. No changes were made to the elements.

5.2.5. MANUSCRIPT V

Manuscript I – IV presents the effects of modulating CPT1 and CPT1A by using etomoxir and the *Cpt1a* P479L mutation in mouse and rat models mimicking ALS-, PD- and MS-like disease. *Manuscript V* (Appendix E) investigates the role of downregulating CPT1 and CPT1A in EAE, SOD1 G93A and rotenone mouse models. It presents that etomoxir ameliorates disease symptoms and diminishes serum inflammatory cytokines in EAE-induced animals and that *Cpt1a* P479l mice are resistant to EAE induction and have decreased serum inflammatory cytokines levels compared to wild type mice induced with EAE [279]. Further, it presents that *Cpt1a* P479L are characterized by a shift towards glucose metabolism based on serum glucose levels and a lower LDL/HDL ratio compared to non-mutated mice [279]. In addition, it presents that *Cpt1a* is epigenetic regulated in BBB-cells obtained from the rat BBB [279]. The manuscript investigates the effect of downregulating CPT1 and CPT1A by etomoxir or heterozygote *Cpt1a* P479L mutation in the SOD1 G93A mouse model. Pilot data are presented indicating that etomoxir and heterozygote *Cpt1a* P479L mutation slows down disease progression based on neuroscore, hangwire, grip strength and gene expression of inflammatory, oxidative stress and mitochondrial biogenesis markers [279]. Furthermore, *manuscript V* presents behavioural motor and sensorimotor data indicating positive effects of downregulating CPT1 and CPT1A in the chronic rotenone mouse model [279], which is substantiated in *manuscript II*. Finally, it presents that *Cpt1a* P479L mutated mice have changes in their gut microbiome, which indicates that CPT1A activity modulates the gut microbiome [279]. Based on these data *manuscript V* present how different neurodegenerative diseases can all be seen from a multisystem perspective based on the systemic platform, as presented in *manuscript V*, and in a more evolved version in this thesis. Even though these diseases have also individual characteristics, they are

alike in the underlying disturbance of the glucose-lipid metabolism balance, thereby identifying CPT1 as a potential relevant target in all of them.

5.3. DOWNREGULATION OF CPT1 AND ITS EFFECTS IN NEURODEGENERATIVE DISEASE MODELS

Manuscript I – V describe the effects of modulating CPT1 activity in multiple *in vivo* models of neurodegenerative diseases. Therefore, this section will summarize the main effects of downregulating CPT1 in the *in vivo* models of neurodegenerative diseases, presented in the manuscripts (**Table 11**).

Table 11: Overview of the mechanisms affected by downregulating CPT1 activity *in vivo* in the manuscripts presented in the PhD thesis

Mechanism	Manuscripts				
	I	**II**	**III**	**IV**	**V**
Shift towards glucose metabolism based on serum glucose levels	x	x			x
Increased mitochondrial biogenesis and possibly decreased mitochondrial dysfunction	x		x		x
Decreased inflammation based on cytokine levels in spinal cord, muscle and serum and gene expression changes in CNS and muscle tissue	x	x		x	x
Decreased oxidative stress based on gene expression	x	x	x		x
Increased myelination/decreased demyelination	x		x		
Possibly increased motor neuron survival	x				
Potential survival of dopaminergic neurons and decreased α-syn levels		x			
Alternations in fecal gut microbiome	x	x			x

- Downregulation of CPT1 results in a shift from CPT1 mediated lipid metabolism towards glucose metabolism based on serum glucose levels in etomoxir treated SOD1 G93A mice (*manuscript I*), SOD1 G93A mice with *Cpt1a* P479L mutations (*manuscript I*). Rotenone exposed wild type mice treated with etomoxir (*manuscript II*), *Cpt1a* P479L mutated mice exposed to rotenone (*manuscript II*) and *Park2* mice treated with etomoxir (*manuscript II*) and *Cpt1a* P479L mutated mice (*manuscript V*).

- Downregulation of CPT1 activity results in increased mitochondrial biogenesis and possibly decreased mitochondrial dysfunction based on increased gene expression of *Pgc1α* in SOD1 G93A mice (*manuscript I*) and EAE mice (*manuscript III*, *manuscript V*). However, further analysis of mitochondrial biogenesis is needed.

- Downregulation of CPT1 activity diminishes inflammation based on lower levels of IL-1β and TNF-α in the spinal cord and tibialis anterior in SOD1 G93A mice treated with etomoxir or *Cpt1a* P479L mutation (*manuscript I*). *Cpt1a* P479L mutation results in lower gene expression of *Iba1* in the midbrain in the chronic rotenone model (*manuscript II*). Etomoxir results in lower gene expression of *Iba1* in the midbrain in *Park2* mice (*manuscript II*). *Cpt1a* P479L mutation results in resistance towards the autoimmune-inflammation mediated EAE-model (*manuscript III*). Etomoxir results in decreased autoantibody recognition of brain antigens in the MBP-induced EAE rat model (*manuscript IV*). Etomoxir and *Cpt1a* P479L mutation results in decreased serum levels of TNF-α and IL-6 in MOG-induced EAE (*manuscript V*). Decreased CPT1 activity due to etomoxir or *Cpt1a* P479L mutation further results in increased gene expression of apoE in SOD1 G93A mice (*manuscript I*) and decreased autoantibody reactivity towards apoE in EAE rats (*manuscript IV*). Increased apoE is associated with decreased inflammation [384] and knockout of apoE results in disruption of the BBB [386]. In addition, downregulation of CPT1 activity results in decreased demyelination in EAE (*manuscript III*) and SOD1 G93A (*manuscript I*). This in agreement with Shriver et al. (2011) and Mørkholt et al. (2020) showing significant effects of downregulating CPT1 activity in EAE mouse models [203,383].

- Decreased CPT1 activity due to etomoxir or *Cpt1a* P479L mutation results in decreased gene expression of oxidative stress and increased expression of oxidative defence markers in SOD1 G93A mice (*manuscript I*), rotenone models (*manuscript II*) and EAE mouse models (*manuscript III, V*). However, further analysis of actual oxidative stress damage is needed.

- Downregulation of CPT1A activity in SOD1 G93A mice increases MNs survival in the spinal cord (*manuscript I*) and decreases denervation in tibialis anterior muscle (*manuscript I*). Accordingly, downregulation of β-oxidation in muscle

tissue in SOD1 G93A mice by ranolazine was recently shown to re-establish muscle glucose metabolism [380].

- Decreased CPT1 activity due to etomoxir or *Cpt1a* P479L mutation results in increased TH, decreased α-syn and increased dopamine in chronic rotenone mouse models (*manuscript II*).

- Downregulation of CPT1 and CPT1A activity modulates the gut microbiome in SOD1 G93A (*manuscript I*), rotenone (*manuscript II*) and *Cpt1a* P479L mice (*manuscript V*).

Additionally, etomoxir has been shown to decrease the level of ferritin in an EAE rat model [203]. Therefore, based on the above summarized results, CPT1 mediated lipid metabolism is indicated to be a potential key target in restoring disrupted homeostasis and amelioration of pathogenic mechanisms due to the overlapping of features between the neurodegenerative diseases like ALS, PD and MS investigated in this PhD thesis (**Figure 11**).

5.4. LIMITATIONS AND FUTURE PERSPECTIVES

The work presented in this thesis have started to clarify the role of dysregulated lipid metabolism and specifically CPT1 in *in vivo* models mimicking some aspects of ALS, PD and MS. Multiple important aspects need to be considered when developing novel pharmaceuticals including the drugs target engagement in the periphery, CNS and the ability to modify the disease. Based on the data presented in *manuscript I – V*, targeting CPT1 by etomoxir indicates that this drug modifies the disease induction and progression in the SOD1 G93A, rotenone and EAE models. Supporting the significant role of CPT1 is the data presented in *manuscript I, II, III and IV* with the *Cpt1a* P479L mutation that indicates protection of developing PD and MS-like disease and delayed progression of ALS-like disease. However, further studies are needed to decipher the underlying mechanisms and therefore this section will shortly present examples of experiments that could clarify more mechanisms and touch upon some general limitations.

5.4.1. IN RELATION TO CPT1 ACTIVITY AND *IN VIVO* MODELS OF NEURODEGENERATIVE DISEASES

To evaluate and dissect the aetiology and pathogenic mechanisms further in the *in vivo* models mimicking some aspects of ALS, PD and MS the following studies could be conducted in the future:

- Longitudinal studies assessing glucose metabolism in the CNS in SOD1 G93A mice treated with etomoxir and SOD1$^{wt/Cpt1a}$, SOD1 G93A$^{Cpt1a/Cpt1a}$ mice, chronic rotenone mouse model and *Park2* and EAE models from pre-onset of disease

possibly through 18-FDG-PET imaging. This would make it possible to evaluate how glucose metabolism is affected during the disease progression and how CPT1 target engagement affects this, as well as where the metabolism shifts (brain, systemic organs (liver, kidney, spleen, etc), blood, gut etc.).

- Longitudinal sampling of serum, feces samples and tissue from animals to evaluate molecular changes over time. At present, a limitation is the fact that it was only possible to assess changes at one time point. E.g. it would be relevant to know how inflammatory cytokines and metabolites are affected during the disease course.

- Metabolomics to analyse possible changes in metabolites in the SOD1 G93A mice treated with etomoxir and SOD1$^{wt/Cpt1a}$, SOD1 G93A$^{Cpt1a/Cpt1a}$ mice, chronic rotenone mouse models and *Park2* and EAE models to establish the effect of downregulating CPT1 activity at a metabolic level. It is at the moment unknown, if metabolites were modified by CPT1 target engagements and if so, which metabolites.

- Transplantation of gut microbiota e.g. from *Cpt1a* P479L mutant mice into SOD1 G93A mice and vice versa to analyse the effects of the *Cpt1a* P479L gut microbiome in the establishment and progression of disease in the SOD1 G93A mouse model and to evaluate whether the gut microbiome from SOD1 G93A mice can induce symptoms in *Cpt1a* P479L mutant mice.

- Transplantation of fecal material from patients with neurodegenerative diseases into *in vivo* models of neurodegenerative diseases to evaluate whether this affects the clinical and molecular progression of the disease. E.g. fecal samples from ALS patients into SOD1 G93A mice and wild type mice. This would be expected if alternations in the gut microbiome actually plays a role in the disease establishment and progression.

- The *Park2* mouse model modulates an early-onset familiar form of PD and therefore it could be relevant to use an α-syn overexpression model to evaluate the effect of targeting CPT1 in a model, which closer resembles sporadic PD. In addition, even though rotenone exposure results in PD in humans, this cause is possibly not the disease-trigger in most PD cases, which could question the translation of findings from the rotenone model into most cases of sporadic PD. Multiple drivers probably needs to be initiated such as mitochondrial dysfunction due to ageing or pesticide exposure, stress and disrupted gut microbiota.

- Cross the *Cpt1a* P479L mutation into the *Park2* mouse model to evaluate whether this have similar effects compared to etomoxir on inflammation, oxidative stress and mitochondrial dysfunction.

5.4.2 IN RELATION TO ETOMOXIR AND CPT1 TARGET ENGAGEMENT

The work presented in this PhD thesis and in the related manuscripts, etomoxir was used as the pharmaceutical compound but some key aspects could be investigated further in the future:

- As the mode of action of etomoxir is downregulation of CPT1 activity it is possible that other derivatives might be promising in targeting neurodegeneration. In addition, there could potential be synergistic effects by combining etomoxir and other drugs used in the treatment of ALS (**Table 3**), PD (**Table 5**) and MS (**Table 7**). Further, multiple neurodegenerative diseases are associated with depression [279] and etomoxir has been indicated to ameliorate depressive symptoms *in vivo* [387].

- Etomoxir has previously been shown to affect the rate of free FAs utilization in the brain [388], which indicates that the compound passes the BBB. However, it would be desirable to evaluate the concentration of etomoxir within the brain following oral and possibly other routes of administration as this affects the bioavailability of the drug.

- In *manuscript I*, *II*, *III* and *V* a dose of 5mg/kg orally administered etomoxir was used. This dose showed clinical-relevant behavioural effects but nonetheless it would be desirable to conduct experiments to evaluate different dosages. Further, downregulation of CPT1 by etomoxir seems to ameliorate or halt the disease progression in the presented *in vivo* models. However, it would be relevant to assess whether the treatment has any effects if the treatment is paused after a period to see whether etomoxir actually is a disease-modifying drug. Especially to evaluate whether glucose metabolism is reestablished, or if other effects like epigenetic regulation prevents this process to be established and continuous medication therefore is needed

In addition, it is important to note that the work presented in this PhD thesis is based on animal models. *In vivo* models are a useful tool to investigate interventions that cannot be conducted in humans due to e.g. ethical reasons [301]. However, multiple anatomic, immune system and metabolic differences exist between humans and rodents [301], which is essential to keep in mind when focusing on the translational perspective of disease aetiology, pathogenesis and the development of therapeutic compounds. Regarding translation, multiple larger transgenic *in vivo* models, e.g. pigs [389], are under development. This will provide an innovative platform to investigate neurodegenerative diseases, and hence targeting their progression.

CHAPTER 6. CONCLUSION

Neurodegenerative diseases constitute a major problem for the society and wealth being for human kind due to the increased life expectancy as no cure is available for the treatment of neurodegenerative diseases such as ALS, PD and MS. The metabolic homeostasis is indicated to shift from glucose to lipid metabolism in the CNS and periphery in neurodegenerative diseases such as ALS, PD and MS.

Therefore, the overall *aim* of this PhD thesis has been to investigate the role of dysregulated metabolism, and to evaluate the effects of targeting the dysregulated metabolism by downregulating CPT1 (by etomoxir and *Cpt1a P479L* mutations) in rodent models mimicking some aspects of ALS, PD and MS. In general, this PhD thesis *hypothesized* that downregulation of CPT1 lipid metabolism would ameliorate or delay progression of clinical disease symptoms in these models in connection with attenuated disease mechanisms such as inflammation, oxidative stress and mitochondrial biogenesis and demyelination whereas upregulation of CPT1 activity would result in exacerbation of the clinical disease phenotype and the disease mechanisms.

In conclusion, the data presented in *manuscript I – V* and in this thesis illustrates the vast complexity of these neurodegenerative diseases and provides data of amelioration of clinical-relevant behaviour by downregulation of CPT1. This ameliorated clinical behaviour is explained by attenuated pathological disease mechanisms such as inflammation, oxidative stress, denervation, neuronal death and increased mitochondrial biogenesis, shifted metabolism towards glucose utilization and modification of the gut microbiota towards a non-dysbiotic direction. In addition, the data and literature presented in this thesis demonstrate how neurodegenerative diseases potentially can be explained based on a systemic platform.

This thesis has focused on ALS, PD and MS but dysregulated metabolism could potentially also play a role in other neurodegenerative diseases such as Huntington’s disease and Alzheimer’s disease.

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