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STUDENT REPORT

PROTEOME ANALYSIS OF APPPS1-21 AND WILD-TYPE MICE – A NOVEL CHRONIC STRESS MODEL

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Abbreviations

Aβ	β-Amyloid
ACTH	Adrenocorticotropic hormone
AD	Alzheimer's Disease
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
AQP-4	Aquaporin-4
ASD	Autism spectrum disorder
BBB	Blood-brain-barrier
CAA	Chloroacetamide
CMS	Chronic mild stress
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAMP	Damage-associated molecular pattern
EA	Ethylacetate
EEG	Electroencephalography
FASP	Filter-aided sample preparation
GFAP	Glial fibrillary acidic protein
HNE	4-hydroxynonenal
IL-6	Interleukin 6
MAC	Membrane attack complex
MCI	Mild cognitive impairment
mtDNA	Mitochondrial DNA
NPD	Neuropsychiatric disturbance
PAMP	Pathogen-associated molecular pattern
PCA	Principal component analysis
PET	Positron emission tomography
PSEN1	Presenilin 1
PTM	Post-translational modification
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
sAPPβ	Soluble peptide APPβ
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TEAB	Tetraethylammonium bromide
TFA	Trifluoroacid
TNF-α	Tumor necrosis factor α
WT	Wild-type

Abstract

Objective: Dementia is a growing problem, especially in the industrial world, because the life expectancy is increasing. At the moment 10-30 % of the world's population above 65 years of age are effected of Alzheimer's disease, with most cases being sporadic. Many studies of Alzheimer's disease have been made, but to date no one have been able to fully explain the different pathological hallmarks, how different factor affect the disease, e.g. stress and sleep deprivation, or why the disease develops in some people. This project aims to investigate the proteomes in hippocampus of both mice with Alzheimer's disease (APPPS1-21 mice) and wild-type mice with and without exposure to chronic stress.

Methods: To create an overview of the proteins in the different test subjects SDS-page was performed. Afterwards was filter-aided samples preparation (FASP) used to prepare the hippocampus samples for label free quantification using a Thermo-Electron QExactive HF-X mass spectrometer (ThermoFisher Scientific). PERSEUS (Max Planck Institute of biochemistry, v1.6.5.0) and PEAKS Studio X (Bioinformatics Solutions Inc.) were used in order to work with the data, make figures, and any statistical analyses, e.g. volcano plots, Hawaii plots, and Principal Component Analysis (PCA).

Results: The results of the SDS-page did not show any differences between the different mice or between test groups. Both volcano plots and the Hawaii plots showed a significant number proteins that have been either upregulated or downregulated in the different test groups, including proteins involved in the circadian cycle, as well as ApoE, APP, and GFAP, which all are suspected as being involved with Alzheimer's disease. The PCA showed no clear groups of proteins involved in the way the results grouped except those involved in the circadian cycle.

Conclusion: The experiments resulted in several interesting results for the research in Alzheimer's disease. ApoE, which has been implicated in the risk of developing familiar Alzheimer's disease, was found significantly increased in APPPS1-21, whereas it was decreased in mice that have been exposed to stress, and in APPPS1-21 + CMS to an extend that it similar to the wild-type mice. Several proteins involved in the circadian cycle was also found to significantly altered in all test groups compared to the healthy wild-type mice, which can be related to the studies of the effect of sleep deprivation on the accumulation of β-amyloid, also in healthy test subjects.

Resume

Formål: Demens er et stigende problem, specielt i den industrielle verden, da den forventede levetid stiger. Lige nu er 10-30 % af verdens befolkning over 65 år ramt af Alzheimers sygdom, som i langt de fleste tilfælde er sporadisk. Der er lavet mange studier omkring Alzheimers sygdom, men indtil videre har det ikke været muligt fuldtud at forklare de forskellige patologiske kendetegn, hvordan forskellige faktorer påvirker hinanden, for eksempel stress og søvnmangel, eller hvorfor nogle mennesker udvikler sygdommen. Dette projekts formål er at undersøge proteomet af mus med Alzheimers sygdom (APPPS1-21 mus) og vildtype mus, med og uden kronisk stress.
Metoder: For at skabe overblik over proteinerne i de forskellige test dyr blev der lavet SDS-page. Efterfølgende blev hippocampus prøverne klargjort til label free quantification på en Thermo-Electron QExactive HF-X mass spectrometer (ThermoFisher Scientific) ved brug af filter-aided samples preparation (FASP). PERSEUS (Max Planck Institute of biochemistry, v1.6.5.0) og PEAKS Studio X (Bioinformatics Solutions Inc.) blev brugt til at bearbejde data, lave figurer og statistiske analyser, herunder volcano plots, Hawaii plots og Principal Component Analysis (PCA).
Resultater: Resultaterne fra SDS-page forsøgene viste ikke nogle tydelige forskelle på de forskellige mus eller mellem de forskellige testgrupper. Både volcano plots og Hawaii plots viste et stort antal proteiner som enten var opregulerede eller nedregulerede i de forskellige testgrupper, inklusiv proteiner involveret i at styre døgnrytmen, samt ApoE, APP og GFAP, som alle er mistænkt for at være involveret i Alzheimers sygdom. PCA undersøgelser viste ikke nogle grupper af proteiner som kunne være årsag til at resultaterne blev grupperet på den måde de gjorde bortset fra de proteiner der er involveret i døgnrytmen.
Konklusion: Resultaterne af disse forsøg viste flere interessante ting i forhold til forskningen i Alzheimers sygdom. ApoE, som er en risikofaktor for at udvikle familiær Alzheimers, var signifikant opreguleret i APPPS1-21, men var nedreguleret i de mus som også have været udsat for kronisk stress, og videre analyser viste at niveauet i APPPS1-21 + CMS var det samme som i vildtype musene. Flere af de proteiner som er involveret i døgnrytmen var signifikant ændret i alle test grupper i sammenligningen med vildtype musene, hvilket også kan relateres til kliniske forsøg af søvnmangels effekt på akkumuleringen af β-amyloid, hvilket også sker i ellers raske mennesker.

Introduction

Alzheimer's disease:

A growing problem in the world is that the population gets older and older. As a natural part of this the brain will also get older, and thereby have an increased risk of developing certain types of diseases due to the degenerative nature of aging. One of the key pathologies is dementia with Alzheimer's disease (AD) being the most common type (50-70 % of all dementia cases[1]), which affects 10-30 % of the world's population above 65 years of age[2]. AD can be divided into a sporadic and familiar form, where the sporadic is responsible for approximately 95 % of the cases[2,3]. This division is done using the patients' medical history and different tests, e.g. gene testing, as these methods can find the different mutations in genes that increases the risk of development of AD[2]. Although there are two subtypes of AD, the pathology cannot be distinguished and is characterized by progressive problems with memory, declining language skills, cognitive alterations, psychiatric disturbances, and in cases symptoms similar to schizophrenia[4]. Taking a look at the pathogenesis of AD on a cellular and molecular level, different markers can be found including plaques containing β-amyloid and neurofibrillary tangles containing hyperphosphorylated tau, which together are specific for AD[2,5–9]. In the beginning of the disease these plaques can mainly be found in the hippocampus and some of the frontotemporal areas, but as the disease progresses the plaques can be found in all areas of the brain[5,6,9–11].

A major problem in dementia and AD is that the patients are not always aware of the problems, so it is often the relatives that brings the person to the doctor and tells about the difficulties with memory etc. As AD progresses the patient will experience increasing difficulties with memory, ultimately also affecting the long-term memory, as well as different psychiatric problems like apathy, aggression, depression-like behavior, anxiety disorders, and personality disorders[5,6], which leads to earlier institutionalization.

Though the cause of AD and the pathophysiology is not fully understood there are many different studies of how different factors could affect and predict the progression of the disease. Some of these studies have led to theories about the involvement of sleep disturbances[10,12–15], stress[8,16,17], different psychiatric diseases[6,18] as well as neuroinflammation[19–21].

Sleep and the glymphatic system

Just like the rest of the body, the brain has to get rid of all the waste products and excess materials, but unlike the rest of the body the brain do not have lymphatic drainage[14,22–24].

Because of this lack of lymphatic drainage several studies of the brain have been made both in animals and human to explain how the brain then gets rid of these substances. So far these studies have led to the theory of the glymphatic system, named after the similar work of the lymphatic system but where the glial cells are responsible for clearing the excess substances from the brain, especially during sleep[14,23,24]. The suggested principle behind the glymphatic system is an influx of cerebrospinal fluid (CSF) into the parenchyma and subsequent efflux to the perivascular space of the veins, from which it is connected to the cervical lymphatic system[14,23]. The astrocytes are suggested to play a crucial role in this clearance as they have aquaporin-4 (AQP-4) channels on their end feet[14], which enables the fluids to pass in and out of the brain. As the fluid passes through the parenchyma the different waste products and excess nutrients are moved along with it, and eventually into the perivascular space and the lymphatic system[14] (see Figure 1).

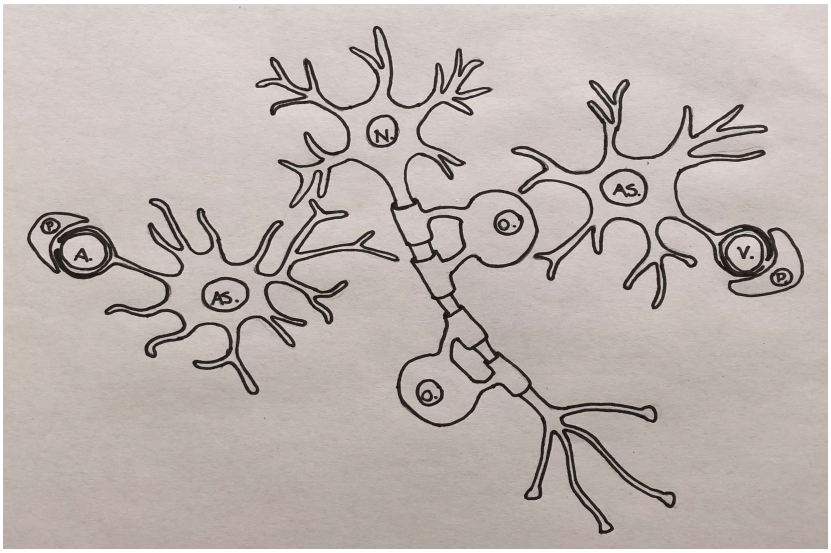


Figure 1: Overview of the components suggested to have a role in the glymphatic system. The system is suggested to work by influx of CSF into the parenchyma by the AQP-4 channels on the astrocytes. The efflux of interstitial fluid happens around the veins, also by the AQP-4 channels. By this movement of fluids any waste products under a certain size can be removed from the brain. The clearance of β-Amyloid is proposed to happen this way and the accumulation inside the brain happens due to oligomerization of the β-Amyloid peptides[14,22–24]. P.: pericyte. A.: artery. AS.: astrocyte. N.: neuron. O.: oligodendrocyte. V.: vein.

Though the system has not been proven yet, strong evidence suggests this system is present in the rodent brain and there are still being done studies in human in order to confirm this system, e.g. to explain the accumulation of β-amyloid in AD.

One of these human studies were performed by *A. Brzecka et al.*[12] whom found the levels of β-amyloid in the CSF decreased with 6 % in the morning when the patients were well rested[12] compared to the levels measured in the evening. Altogether, these studies suggest that any alteration in the function of the glymphatic system might be involved in the development and progression of AD, as one of the important pathogenic hallmarks is the accumulation of the β-amyloid protein,

which appears to be cleared during sleep by this system[10,12,14,23]. If patients suffer from sleep disturbances, the glymphatic system might be compromised and thus the clearance of β-amyloid, which allows an accumulation and over time formation of plaques[10,12,14,23].

N. Volkow et al.[10] made a study of the effects of sleep deprivation compared to normal sleep in healthy subjects. The tests performed included a visual attention experiment, where the subjects has to keep track of moving balls on a screen[25], as well as self-reports of alertness and for the sleep-control also the quality of their sleep. The results showed a worse performance when the patients were sleep deprived compared to well rested controls. They also found an increase in dopamine in different areas of the brain, e.g. the thalamus, after a night of sleep deprivation compared to being well rested[10]. As a part of the analysis of the dopamine system *N. Volkow et al.* looked into the availability of the D2-receptor and found a significant lower availability in people who were sleep deprived compared to well rested[10]. These results were interpreted by *N. Volkow et al.* as a sign of cognitive decline in sleep deprived patients and that an increase in dopamine might be related to cognitive performance[10].

A recent study by *B. Lucey et al.*[26] has also been looking into the relationship between sleep and the levels of different lengths of β-amyloid (β-amyloid$_{38-42}$)[26]. Their results showed that the levels of all investigated lengths of β-amyloid in the CSF were increased in patients that had been sleep deprived compared to both controls and induced sleep. This was done using sodium oxybate that is known to induce deep sleep, which led the investigators to the conclusion that the clearance is not affected, as the fractural turnover rate is not changed, but the activity of β-secretase is increased during sleep deprivation[26]. This might be due to an increased activity of the neurons during wakefulness as well as a decreased glymphatic clearance at the same time[26].

Stress

Stress can be categorized into physiological and psychological stress, where the physiological stress is a reaction to e.g. a threat and the psychological stress is a reaction to e.g. performance on the job or in school. Stress in general has been linked to the risk of developing AD[3,16,17,27–33], and several studies have been made to test the effect of stress on the body. In rodents, different stressors have been investigated, and the effect on cognition and learning have been measured by using different types of mazes[27,28,32–34]. A few studies have also been performed on human volunteers. One of these studies were performed by *L. Johansson et al.*[17] where women were followed for 35 years with four follow-ups, including questionnaires and medical examinations, to look for relations between the

women's self-reported stress levels and the risk of developed AD[17]. The investigators found a positive correlation between stress levels and the risk of developing of AD[17].
Other studies looked at stress and neurodegeneration in comparison to normal brain aging to understand the involvement of stress on normal aging and development of neurodegenerative disorders[3,16,29–31,35]. One of these studies performed by *S. Vyas et al.*[35] investigated the effect of the elevated levels of glucocorticoids[8,12,16,17] in relation to developing dementia, and found that the elevated levels of glucocorticoids alters the brain plasticity, which can lead to neurodegeneration[35], as the brain no longer is able to form new connections if needed.

Neuropsychiatric disturbances

AD is not only characterized by cognitive impairment, but also by neuropsychiatric disturbances (NPDs) including depression-like behavior and anxiety. Why these disturbances occur together with AD is not known, but one hypothesis is an increased susceptibility in AD patients compared to the otherwise healthy elderly population. One of the areas linked with both anxiety and depression as well as AD is the limbic system, e.g. the amygdala and hippocampus. Due to the degeneration of these areas in AD, it might be the explanation of some of the symptoms these patients experience. As the NPDs in AD are diagnosed based on occurrence and frequency of symptoms (the Neuropsychiatric Inventory), several studies have been performed to identify specific biomarkers[36–39]. Some of these studies have looked at the morphology of the different cells in the brain and found a link between the morphology of dendrites and autism spectrum disorders (ASD), schizophrenia, epilepsy[39] and AD[36]. In the study by *P. Penzes et el.*[36] they found that ASD was associated with increased dendritic density, maybe because of a faulty pruning, and both schizophrenia and AD was associated with a decreased density, though at different time points in life[36]. *M. Toepper*[37] compared normal brain aging to the AD brain and found that the age-related changes in the grey matter was opposite in the AD patients compared to healthy aging controls. The AD samples showed an increased degeneration in hippocampus, insula, putamen, nucleus caudatus, as well as areas of lobus frontalis[37]. The study also found the white matter to be most affected in the areas with degeneration of the grey matter[37]. These changes in the dendritic density in AD has also been linked to the increased risk of epileptic seizures, as there might be a decrease in both inhibitory and synchronizing signals[39], especially in patients with the familiar form of AD[37], and might even be a possible way to identify people at risk of developing AD later in life[37].

A lot of studies using electroencephalography (EEG) have also been made in order to identify any activity related explanations for the relationships and similarities of different types of neurological issues[37–40]. These studies have found some distinct differences in EEGs in AD patients compared to healthy age-matched controls. The normal EEG patterns during specific tasks were found to be altered in patients with mild cognitive impairment (MCI) and AD compared to the healthy controls, where the areas that should have a downregulated activity showed an increased activity, which might explain the increased risk of epilepsy in patients with dementia. The activity patterns have also been linked to progression and severity of AD[39,40].

These dysregulated EEG patterns in MCI and AD also showed a close association with the β-amyloid deposition[39]. *F. R. Kurudenkandy et al.*[38] investigated the relation between β-amyloid and EEG changes. They found that the β-amyloid disrupted the gamma-waves in the EEG, and that this disruption was dependent on the state of the β-amyloid (monomeric, oligomeric or insoluble) with the latter having a larger effect on EEG patterns. They also tested if this toxic effect could be prevented using two different chaperones, and found that these effects could at least partially be prevented[38]. The results of that particular experiment shows that this area might be very important in regard to treatment of AD, as such compounds might be able to stop the progression into more severe cases of AD.

Held together these studies found desynchrony in different areas of the brain, which resulted in epilepsy-like seizures and lack of deactivation respectively[37–40]. A possible reason for this desynchrony of brain activity in AD patients could be explained by the β-amyloid accumulation, which compromises the function of synapses and dendrites[41]. If enough dendrites and synapses are destroyed the normal signaling network will be disrupted, and might be the cause of the differences in synchrony in the AD, and therefore might be a possible biomarker for developing MCI and the progression into AD.

Neuroinflammation and glial cells

A consistent finding throughout patients with neurodegenerative disorders is neuroinflammation, although the exact mechanisms has not been established[19–21,42–44].

In the central nervous system (CNS) there is only one type of immune cells, the microglia, which are macrophage-like[21], but the other types of immune cells are normally not able to pass through the blood-brain-barrier (BBB) as they are too big to pass through the tight-junctions[29]. The microglia are able to recognize pathogen-associated molecular patterns (PAMPs) as well as damage-

associated molecular patterns (DAMPs) and thereby activate the complement system[19–21]. Some of the known activators are β-amyloid and cell debris after the death of cells in CNS, which are known pathological hallmarks of AD[19–21]. *G. Brown and J. Neher*[45] studied the phagocytosis by microglia and how it could affect the brain. Their results showed that the microglia are not only able to phagocytose the dying or dead cells, but also living neurons if they express stress signals, e.g. expression of the phospholipid phosphatidylserine[45], which might explain the neurodegeneration in AD.

Another important cell type in the CNS is the astrocytes, which play a role in maintaining brain homeostasis, and many studies have been made about changes in AD mice brain compared to wild-type (WT) controls[21,46–49]. These studies shows that astrocytes in AD compromised brains do not degenerate, but their morphology change[46–49]. The astrocyte studies found that there is a general astrocytic atrophy in the brain areas with a low amyloid burden, whereas the areas with a high burden have hypertrophic astrocytes, which indicates the astrocytes are activated by the β-amyloid plaques[46–49]. Another involvement of the astrocytes might be due to their role in glutamine synthesis for the neuron's glutaminergic signals[48]. *M. Olabarria et al.*[48] studied the glutamine synthase in astrocytes from AD transgenic mice. Their results showed a decrease in the expression of glutamine synthase and glial fibrillary acidic protein (GFAP) in the transgenic mice brain compared to a WT control[48]. The investigators concluded that this decreased expression would lead to a decreased conversion of glutamate to glutamine, which thereby could exceed the toxic levels of the neurotransmitter[48]. This might be a crucial part of the pathogenesis of not only AD but also Parkinson's disease, as the disruption of the neurotransmitter levels might have excitotoxic effects which can exacerbate the neurodegeneration.

Both *M. Heneka et al.*[19] and *N. Working et al.*[20] have reviewed neuroinflammation and the involvement in neurodegenerative disorders like AD. They reported that the β-amyloid is able to activate the complement system, possibly via the oligodendrocytes[42], and thereby make membrane attack complexes (MACs) to form in the dendrites[19,20], which might be related to the dendritic atrophy in neurodegenerative diseases as reported by *P. Penzes et al.*[36]. Besides the complement activation there will also be an increased pro-inflammatory stimulation of the cells in the brain by e.g. releasing interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α)[19]. *M. Heneka et al.*[19] also reported that IL-6 is able to increase the production of adrenocorticotropic hormone (ACTH), which stimulates the adrenal glands to produce glucocorticoids[19] and in turn leads to increased levels of stress.

Oxidative and nitrosative stress

A pathological hallmark that is thought also to have a major role in the development and progression of AD is an increased oxidative and nitrosative stress[43,44,50–54]. Studies of the causes of the increased stress as well as the effects have been made, and results suggests that post-translational modifications (PTMs) and accumulation of different redox active metals affects the cell's normal function by creation of misfolded proteins[43,44,50–54]. *D. Butterfield et al.*[50] investigated nitration and its role in the development and progression of AD. The results showed that a high number of nitrations correlated with advanced AD symptoms compared to MCI and healthy controls[50]. They also suggested that the nitration could affect the folding of proteins by inhibiting other types of PTMs, e.g. phosphorylation, as it would happen at the same site[50]. *M. Heneka et al.*[19] have also studied the effects of nitration, and their review suggests that nitration of β-amyloid inhibits the ability to form long-term potentiation compared to a non-modified β-amyloid[19]. Furthermore the study also showed that the nitrated β-amyloid is more prone to form plaques than other forms of β-amyloid[19].

The important aspect of the increased oxidative and nitrosative stress is the accumulation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) which are able to form free radicals[43,44,53,54] from the accumulated metal ions, especially iron which reacts via the Fenton and Haber-Weiss reactions (see Figure 2)[54].

$$Fe^{3+} + O_2 \cdot^- \leftrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \leftrightarrow Fe^{3+} + HO \cdot + OH^- \text{ (Fenton reaction)}$$

$$O_2 \cdot^- + H_2O_2 \leftrightarrow O_2 + HO \cdot + OH^- \text{ (Haber Weiss reaction)}$$

Figure 2: The Fenton and Haber Weiss reactions showing the reactions of either ferrous or ferric iron in the formation of free radicals. The first line shows the relation between the two different oxidative states of iron, and how they can be formed in the presence of reactive oxygen. The second line shows the Fenton reactions where the ferrous iron reacts with hydrogen peroxide to form ferric iron, a hydroxyl radical, and hydroxide. The Haber Weiss reaction shows the total reaction from the two other lines. The iron is not shown in this equation as it is preserved. The figure is modified from W. Markesbery[55].

A major problem with the formation of RNS, ROS, and the free radicals is they cause major damage to both RNA and the mitochondrial DNA (mtDNA)[56–58]. *P. Moreira et al.*[57] studied the mitochondria and the dysfunction as a trigger of AD. Their results showed that the mitochondria are highly susceptible to the oxidative damage and that the damage was worse in lobus frontalis in the AD samples[57].

A genetic risk factor linked to the development of AD is the apolipoprotein E (ApoE) 4 allele[4,11,59–61]. One of the normal roles of ApoE is to bind redox active metals and thereby have a protective role of the cells, and especially the highly vulnerable mitochondria. The most common form of

ApoE is the ApoE3 allele, with ApoE2 and ApoE4 less common[4]. Patients with homozygotic ApoE4 have up to a tenfold increased risk of developing AD, as well as developing it earlier compared to the other genotypes[59]. ApoE studies have also shown that it is involved in the production of ROS and is involved in apoptosis[62] as well as binding[63] and breakdown[62] of β-amyloid.

These results suggests that the ApoE genotype might have a vital role in the accumulation of metal ions, oxidative and nitrosative stress, and the accumulation of β-amyloid and plaques.

Disease theories

Though the exact pathophysiology of AD is not fully understood, the growing number of studies of AD has led to a number of hypotheses for the development and progression rate of AD. One of the most prominent hypotheses about AD is called the β-amyloid cascade theory[6,39,40,42], which focuses on the role of β-amyloid accumulation. Under normal conditions the amyloid precursor protein (APP) is cleaved by α-secretase and afterwards by γ-secretase, which leaves the α-amyloid peptide, but for an unknown reason this can shift to a cleavage made by β-secretase and then γ-secretase[2,6,39–42,59,64,65] (see Figure 3). This way β-amyloid is produced and can over time aggregate with other β-amyloid peptides in order to form polymers and eventually plaques[39,40]. As the production of β-amyloid in the AD brain is higher than in a healthy brain only some of it is cleared and leaves the rest to disrupt the neural signaling[20], which accelerates as oligomers and plaques are formed[38].

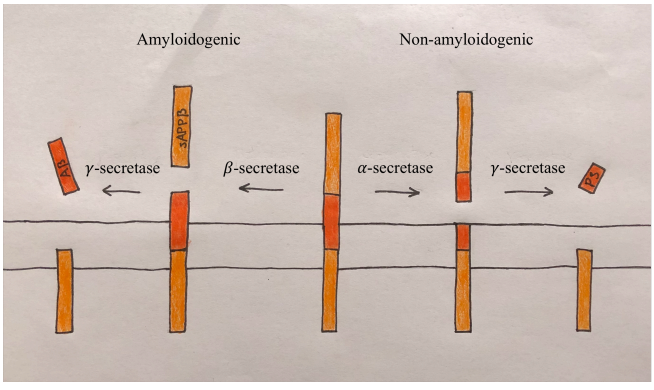


Figure 3: The two types of processing of APP. The right side shows the non-amyloidogenic process in which α-secretase and γ-secretase are cleaving APP resulting in the P3 peptide. The left side shows the amyloidogenic process in which β-amyloid is created by a cleaving of APP by β-secretase and γ-secretase[2,6,39–42,59,64,65]. sAPPβ: soluble peptide APPβ. Aβ: β-Amyloid.

F. Kurudenkandy et al.[38] studied the different forms of β-amyloid in order to find out which form is the most toxic to the brain from an EEG point of view. The researchers found that the gamma

waves were decreased in monomeric β-amyloid compared to the control, a bit more in a mixture of different forms of β-amyloid, and most in the β-amyloid fibrils. They also found that the exposure time was significant, with a longer exposure time resulting in a greater decrease in gamma waves[38]. In general these results suggests that the polymerization of the β-amyloid peptides increases the toxicity of it.

Another hypothesis is related to the importance of interneurons and the normal signaling between different areas of the brain. Studies of the brain signaling found that the interneurons in ASD, schizophrenia, epilepsy, and AD all were altered compared to healthy controls[37–40], and this might be related to the memory impairment as well as the personality changes seen in AD patients. These disruptions of networks also showed as hypersynchrony and desynchrony in different areas of the brain[37–40], which means that e.g. the normal inhibitory signaling will be disrupted too which can lead to excitotoxicity and thereby also cell death and subsequently inflammation. *J. J. Palop and L. Mucke*[39] looked into the relationship between the altered activity patterns in the brain and MCI, where they tried using an anti-epileptic drug (Levetiracetam) in low doses, and found that it was able to normalize the activity in hippocampus after 2 weeks of treatment and improved the performance compared to the baseline[39]. Taken together with a lot of failed clinical trials for treating AD the results from *J. J. Palop and L. Mucke*[39] indicates there might be a limited window for treatment, as the effects in MCI can be reverted but not in the patients with advanced stages or AD. If the treatment options indeed are limited to a specific time in the disease progression, the need for biomarkers and early diagnostic tools is increasing, as the patients have to be identified at a very early stage where the plaques and tauopathies might not be detectable using e.g. positron emission tomography (PET) scanning, which is normally used in order to investigate the plaque burden in AD patients. Another important part of the pathogenesis is that hippocampus is one of the first areas to be hit by the β-amyloid accumulation in AD[2]. According to both *J. J. Palop and L. Mucke*[39] and *R. Verwer et al.*[66] there is a relation between the activity of the different brain areas and the progression of plaque distribution, which is also shown in Figure 1 in the review by *C. Masters et al.*[2] and Fig. 1 in *D. Van Dam et al.*[6]. Hippocampus has a high metabolic activity, and high metabolic activity is linked to the risk of accumulating β-amyloid, which might be an explanation for this area to be affected early in AD[66]. Together with the hippocampal hyperactivity in MCI patients[39] this might be a possible target for future research into both the driving forces of AD progression and the possible areas for therapeutic interventions[39].

Another area that could be investigated more is the ratio between two types of β-amyloid linked to AD, β-amyloid$_{40}$ and β-amyloid$_{42}$, as the ratio between these two appear to change in MCI and AD, and the different lengths of the peptide have different tendencies to form oligomers[38,67,68]. This area might also be an area for future treatment research, as a difference in ratios of β-amyloid$_{40}$ and β-amyloid$_{42}$ are seen in MCI and AD and if the balance can be restored it might alleviate some symptoms or reverse the plaque deposition.

Along with the β-amyloid plaque formation the neurofibrillary tangles are very important in the AD pathology[8,27,69]. In healthy cells the tau protein is crucial for the structure of the microtubules[69], but in the case of AD the protein gets hyperphosphorylated and thereby loses its function[8,27,67,69]. This leads to collapse of the neuronal morphology and the formation of neurofibrillary tangles[8,27,69].

Modelling AD in rodents

As many diseases, including AD, have a pathology that is not understood, a way to investigate them under specific conditions is to make animal models of the diseases, though it might give rise to translational problems. In some studies of the inflammatory response in the neurodegenerative disorders there have been some problems in the translation from animal models to human subjects, as some of the cytokines released are protective in rodents but are destructive in human, e.g. TNF-α which both have a proinflammatory and anti-inflammatory property[37–39], but only rodents had a benefitial effect in relation to neurodegeneration, whereas the situation got worse in human[20].

There are both *in vitro* and *in vivo* systems and they can be used to investigate different aspects, and both types of studies have their advantages and disadvantages. *In vitro* studies are very common in the research of individual cell types and how they react, whereas the *in vivo* studies are used in research of how different organs or organ systems reacts in a given condition, e.g. in AD.

A major advantage of using animal models is the ability to make transgenic models to mimic the human pathology. According to AlzForum.org[70] there are, at the moment, 168 animal models for the research of AD, which have different mutations and combinations of pathologic hallmarks. In order to create these transgenic models specific mutations of genes can be knocked in by injecting them into the male pronuclei of an oocyte[67]. Afterwards the individuals carrying the specific mutations can be used for further breeding, e.g. by mixing it with another transgenic line. In this way an animal model with different mutations and pathologic hallmarks can be made, e.g. the APPPS1-21 mouse, which is used for this project. These mice have human mutations in APP and

presenilin 1 (PSEN 1) which both are associated with development of early onset AD, and the mice start showing amyloidosis in the cortex at 6-8 weeks of age[67].

Another way to model AD in rodents is to use the fairly new TgF344-AD rat. Similar to the APPPS1 mouse this rat has human mutations in the APP and PSEN1 genes, but because the rat is closer related to the human the new model is being investigated[71]. *J. Michael et al*.[71] investigated TgF344-AD rats and WT rats at a course of 18 months to compare differences in the transgenic model compared with healthy age-matched controls. This study found that dysfunctions of the connectivity in the brains of the transgenic rats was detectable before any morphological changes or formation of amyloid plaques, and that these changes were not detected in the WT rats[71].

Project background and aim

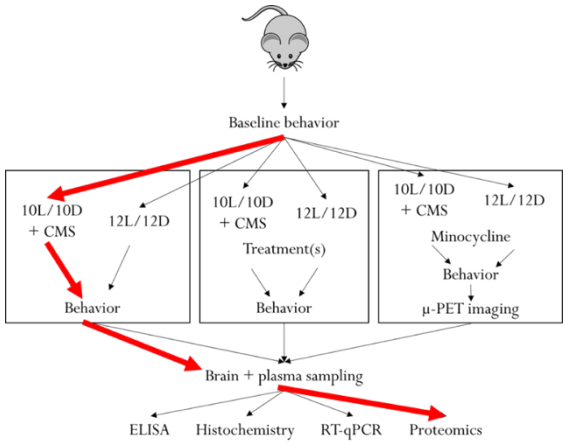
This master thesis has its background in a PhD project called "*Characterisation and Modulation of Neuropsychiatric Disturbances (NPD) in a Preclinical Mouse Model of Alzheimer's Disease*", which is currently being made at H. Lundbeck A/S. The aim of the PhD project is to investigate different aspects of using the murine AD model, APPPS1-21, for investigation of neuropsychiatric disturbances cooccurring with AD. The first part of the PhD project was to investigate behavior and genes in WT and APPPS1-21 mice before and after the exposure to chronic mild stress (CMS). The results of these test showed an increase in rearing and anxiety-like behavior of the mice. In relation to the genes the tests showed changes in the genes controlling the circadian clock. A part of this test also included testing of the activity levels of the mice during the light and dark phases. In the non-stress mice there was a normal activity pattern with most activity during the dark phase, but in the stressed individuals this clear difference was not present.

This current project is part of the second part of the PhD project, which is to investigate and evaluate proteome analysis to study NPD pathology and novel biomarkers of the mice enrolled in the behavioral studies. In this way it is possible to investigate any biological explanation for the behavior seen in the mice. A proteome strategy was investigated including multiple optimization steps, including choice of tissue lysis, choice of mass spectrometry based quantitation strategy, and data mining. A robust lysis method based on mass spectrometry compatible lysis buffer, visualization by SDS-page analysis to create an overview of the protein content in the samples, and a label free quantification in order to enable the direct comparison of proteome PTMs in the different groups of mice. Only the final data have been included in this report.

Methods

Animals

Two types of mice were used for the experiments, WT and APPPS1-21 (n = 10 per group, total n = 40). Both types of mice were divided into two groups, one for control and one for chronic mild stress. After behavioral tests the mice were decapitated and their brains removed, with immediate removal of the hippocampus, and stored at -80° C.



These procedures were performed at H. Lundbeck A/S and were approved by the National Committee on Health Research Ethics.

Tissue lysis and protein content

The hippocampi were divided into two equally sized parts where one is used for the test and the other part saved for future tests. The hippocampi were placed in separate tubes containing 100 µL 5 % sodium deoxycholate (SDC) and small 0.4 - 1.2 mm stainless beads. The samples are then homogenized by bead beating for 10 min at maximum speed (Next Advanced; Bulletblender GOLD).

The protein content were afterwards determined using a Protein A280 protein quantification (Thermo Sci Nano-drop), and the mean value from three tests was used for the subsequent experiments (see overview in appendix A).

Another analysis was performed after the sample preparation (see overview in appendix A).

Visualization of tissue lysis by SDS-page

The samples are prepared by mixing 1:4 Laemmli Sample buffer (Bio-Rad, 161-0747), 1:10 Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Sigma-Aldrich, C4706), MQ-water and 10 µg protein. After a thorough mix the samples are boiled for 5 minutes at approximately 95° C.

Five 4-15 % Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, 456-1083) are prepared and placed in the chambers, which are filled with running buffer (Tris base, glycine, SDS, and MQ water). 5 µL PageRuler™ Unstained Broad Range Protein Ladder (Thermo Scientific, 26630) were loaded in the first wells and 20 µL sample were loaded in the subsequent wells (see setup in table 1). The gels are running for approximately 45 minutes and the removed from the cassettes for Krypton staining.

First the gels were incubated twice at 30 minutes in fixing buffer (40 % ethanol, 10 % acetic acid, and MQ-water). The gels were washed for 5 minutes with MQ-water before incubation in Krypton™ Fluorescent Protein Stain (Thermo Scientific, 46630) for 1h. After the staining the gels were soaked in destain (5 % acetic acid) for 5 minutes and then washed twice in MQ-water for 15 minutes. The gels were scanned using a Typhoon 9410 (Variable Mode Imager) scanner.

Table 1: Setup for SDS-page. Five gels were prepared in order to have room for all 40 samples. Each gel has a ladder in the first well and then loaded with 2 samples from each group of mice. The numbers refer to the animal facility ID of the mice..

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	Well 10
Gel I	Ladder	33	82	25	86		34	83	26	87
Gel II	Ladder	35	84	31	88		36	90	32	89
Gel III	Ladder	37	95	39	91	38	96	40	92	
Gel IV	Ladder	41	97	44	93		42	98	45	94
Gel V	Ladder	43	100	46	99		49	101	47	102

Sample preparation

For preparation of the samples for mass spectrometry analysis filter-aided sample preparation (FASP) was used. 100 µg protein was transferred to spin tubes with a flat filter. 0.5 % SDC was added in order to reach a total volume of 200 µL in the tube. The tubes are centrifuged at 14,000g for 15 minutes, and the flow-through is discarded. 200 µL digestion buffer (0.5 % SDC in 50 mM tetraethylammonium bromide (TEAB)) is added together with 1:50 TCEP and 1:20 chloroacetamide (CAA) and the samples are incubated for 30 minutes at 37° C. Samples are centrifuged at 14,000g for 15 minutes again, and the flow-through is discarded. Another 200 µL digestion buffer is added and centrifuged 14,000g for 15 minutes. 2 µg trypsin is added to all tubes, which are incubated at 37° C the night over.

The samples are centrifuged at 14,000g for 15 minutes. Afterwards 100 µL 50 mM TEAB is added, and the samples are centrifuged for another 15 minutes at 14,000g and then the filters are discarded. To remove the SDC from the peptides a phase separation is made. This is done by adding 150 µL ethylacetate (EA) with 1 % trifluoroacid (TFA). The samples are then vortexed and centrifuged for 1 minute at 14,000g. The upper organic phase can then be removed. Another 150 µL EA, this time without TFA, is added and another centrifugation is done. The upper phase is removed again. The samples are lastly vacuum centrifuged in order to dry them.

The samples are now ready to be resuspended and labeling for the mass spectrometry analysis.

Label free quantification

2 µg of each sample (n = 40) were loaded in a randomized order in a well-plate. The samples were loaded onto a reversed phase column (Dionex; Acclaim PepMap100 C18,
5 µm precolumn and 75 cm Acclaim Pepmap RSLC, 75 µm ID main column, Thermo Scientific) and eluted with a linear gradient of 96% solvent A (1% formic acid) and 4% solvent B (acetonitrile) increasing solvent B to 35% on a 60 min ramp gradient.
The samples were analyzed using an automated liquid chromatography-electrospray ionization MS/MS system with a Dionex ultra performance liquid chromatography system. The system is coupled to an emitter for nanospray ionization into a Thermo-Electron QExactive HF-X mass spectrometer (ThermoFisher Scientific).

Data analysis

For analysis of the data from the label free quantification PERSEUS (Max Planck Institute of biochemistry, v1.6.5.0) was used. The raw data file from the mass spectrometer is loaded, and through multiple steps data has been refined (see workflow in Figure 4). Before exclusion of data points all data was log2 transformed. In order to be able to do a Principal Component Analysis (PCA) missing values were interpolated assuming the data is normal distributed using standard parameters in PERSEUS (width = 0.3, downshift = 1.8). Technical replicates were combined by the mean, and differentially expressed proteins (DEPs) were identified by t-tests. The data was corrected for multiple hypothesis testing using permutation-based false-positive control with standard parameters in Perseus (s0 = 0.1, FDR < 0.05). Protein function was analyzed using Gene Ontology nomenclature from UniProt protein knowledgebase (UniProtKB) annotation. Unsupervised hierarchical clustering with Euclidean distance calculations was performed on z-score normalized data using standard parameters in Perseus (300 clusters, 10 iterations). Data were also searched using PEAKS Studio X (Bioinformatics Solutions Inc.) in order to investigate PTMs.

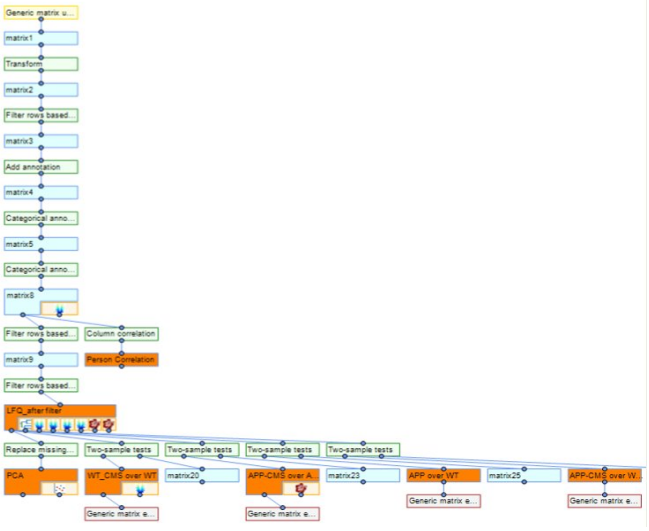


Figure 4: Workflow for refining the data from label free quantification

Results

Evaluation of tissue lysis by protein A280

In order to validate the protein extraction and digestion into peptides a protein quantification by protein A280 by Nano-drop analyses were performed, which are shown in Appendix A. These results were also used in order to make SDS-page and quantitative MS analyses concentration dependent.

Visualization of tissue lysis by SDS-page

Poly acrylamide gel electrophoresis (SDS-page) was performed in order to visualize the protein content and their sizes in the hippocampus samples. In order to analyze all 40 samples five gels were run, each containing a ladder and two samples from each test group (see Figures 5-9). Generally, the results have no major differences between the different test groups or samples. There are many proteins with low mass detected, indicated by the abundance of bands in the bottom of the gels, compared to the top of them, where the proteins with a high mass is located. A minor difference is found in mouse 87 (WT), where two bands between approximately 50 and 60 kDa have been detected, which is not detected in the other samples. Whether this is a genetic variance or has some other reason cannot be determined from these experiments, but maybe from the subsequent experiments. There are also small differences in the intensity of bands on the gels, which may be due to different expression of the proteins or small differences in the proteins extracted prior to the experiment.

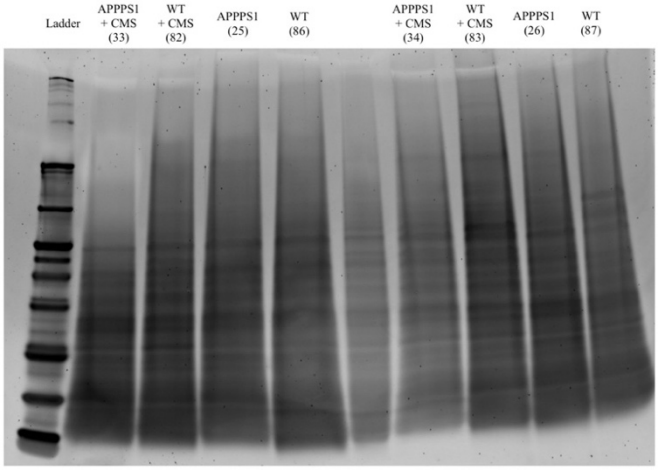


Figure 5: Representative gel 1 containing a ladder and hippocampus lysates from mouse 33, 82, 25, 86, 34, 83, 26, and 87. The lanes have been marked with their respective test group and ID. No major differences can be detected across the different samples, though there are two bands detected at 50-60 kDa in sample 87 that cannot be seen in the other samples.

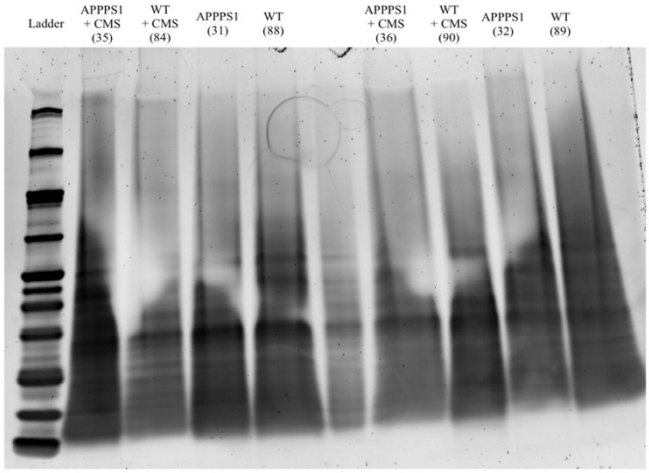


Figure 6: Representative gel 2 containing a ladder and hippocampus lysates from mouse 35, 84, 31, 88, 36, 90, 32, and 89. The lanes have been marked with their respective test group and ID. No major differences can be detected across the different samples. The appearance of the gel is very different from the others, but the reason could not be determined.

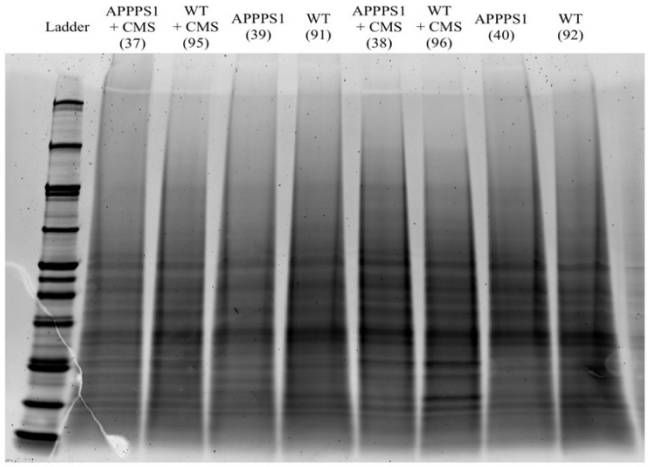


Figure 7: Representative gel 3 containing a ladder and hippocampus lysates from mouse 37, 95, 39, 91, 38, 96, 40, and 92. The lanes have been marked with their respective test group and ID. No major differences can be detected across the different samples.

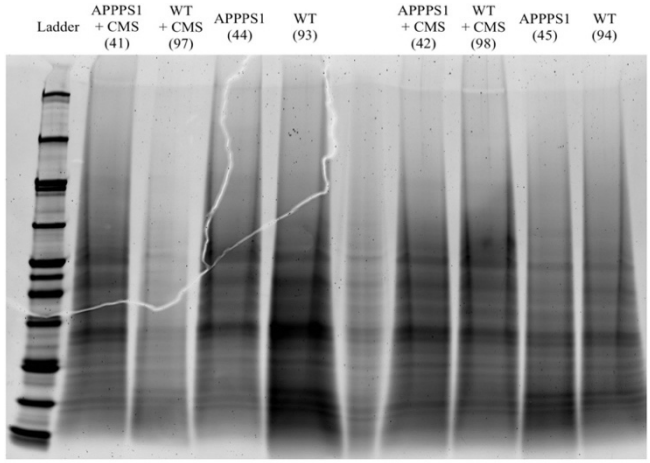


Figure 8: Representative gel 4 containing a ladder and hippocampus lysates from mouse 41, 97, 44, 93, 42, 98, 45, and 94. The lanes have been marked with their respective test group and ID. No major differences can be detected across the different samples.

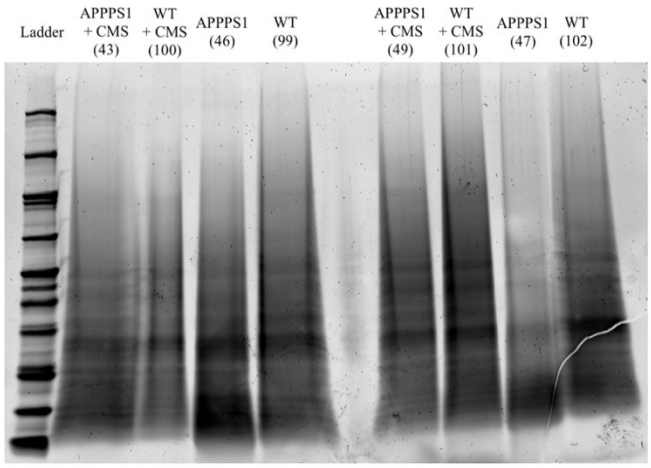


Figure 9: Representative gel 5 containing a ladder and hippocampus lysates from mouse 43, 100, 46, 99, 49, 101, 47, and 102. The lanes have been marked with their respective test group and ID. No major differences can be detected across the different samples.

In order to investigate the proteins further a label free quantification was performed. This analysis was chosen in order to investigate the proteins in each test subject and compare them across test groups.

Mass spectrometry based Label free quantification of proteome

In order to evaluate and correlate the CMS to the proteome and individual relative protein content across the different test groups a label free quantification was performed. In order to visualize the data volcano plots (relative quantification vs. p-value), Hawaii plots (multiple Volcano plot with a common denominator), and Principle component analysis (PCA) were performed. As a part of the PCA a protein network analysis by EMBL STRING analyses were performed in order to investigate proteins responsible for the groupings found in the plots of the different components.
A cluster analysis was also performed, but there were no formation of clusters in the data (data not shown).

Volcano plots:

A total of four volcano plots were made in order to visualize all the statistically significant changes in proteins levels, as shown in Figures 10-13. The figures shows all statistically significant alterations above the two lines, with upregulations on the right-hand side and downregulations on the left-hand side (p-value ≤ 0.05). The rest of the dots represent proteins that are not significantly altered. The figures compare two different test groups each time in order to visualize the importance of chronic stress and the mutations in the APPPS1-21 group.

The first volcano plot (Figure 10) shows the comparison of the WT + CMS and WT mice. This comparison shows differences in healthy subjects and otherwise healthy subjects exposed to chronic stress. Looking into the different dots in Perseus, both a lot of the up- and downregulated proteins are related to stress, e.g. ApoE which is significantly lower in the WT + CMS group.

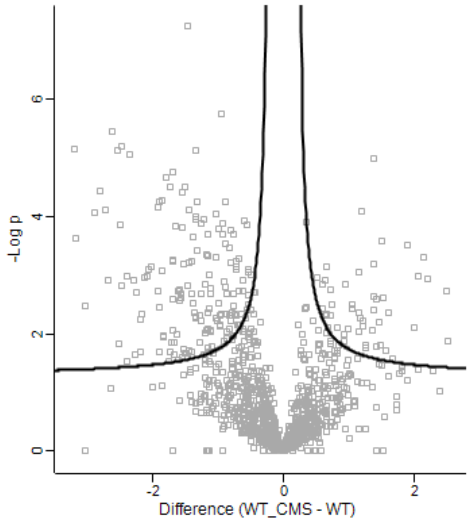


Figure 10: Volcano plot of the differences between the WT + CMS and WT mice. The upper right-hand part of the plot shows all the proteins that are significantly upregulated in the WT + CMS samples compared to the WT samples. The upper left-hand side shows the downregulated proteins. All dots in the center and the bottom are proteins that are not statistically different.

The second volcano plot (Figure 11) shows the comparison of APPPS1-21 and WT mice. Looking through the proteins involved with AD, both ApoE and GFAP shows up as being upregulated in the transgenic mice compared to WT. Though APP is involved in the pathology of AD there was not found any significant difference between the transgenic and WT mice in this experiment.
Selecting the area with downregulated proteins, only a few proteins have been related to AD whereas the rest are related to cellular physiology and regulation.
The rest of the proteins related to AD are not statistically different between the these two groups.

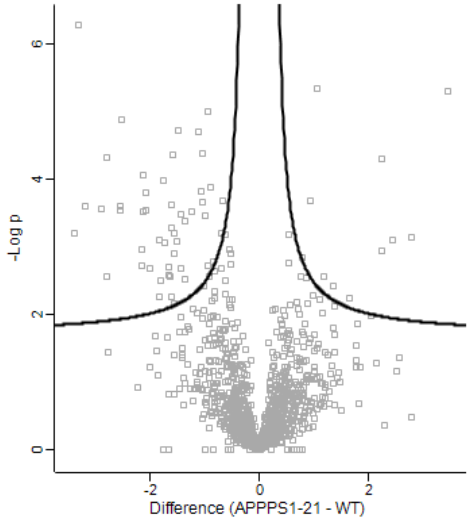


Figure 11: Volcano plot of the differences between APPPS1-21 and WT mice. The upper right-hand part of the plot shows all the proteins that are significantly upregulated in the APPPS1-21 samples compared to the WT samples. The upper left-hand side shows the downregulated proteins. All dots in the center and the bottom are proteins that are not statistically different.

The third volcano plot (Figure 12) shows the comparison of APPPS1-21 + CMS and APPPS1-21 mice. This comparison is important for estimating if chronic stress can worsen the AD. Looking at the significant results by gene onthology and disease relations a lot of the proteins are related to AD, but also the cell physiology, cell cycle, and immunology. Compared with the above-mentioned results, the upregulation of ApoE and GFAP in the mice do not occur if they are exposed to chronic stress, but APP is upregulated in the stressed subjects.

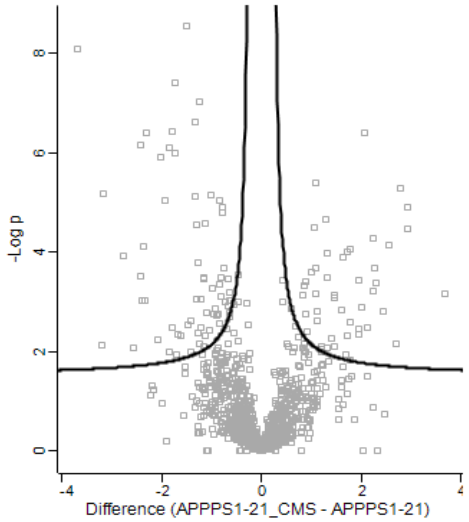


Figure 12: Volcano plot of the differences between APPPS1-21 + CMS and APPPS1-21 mice. The upper right-hand part of the plot shows all the proteins that are significantly upregulated in the APPPS1-21 + CMS samples compared to the APPPS1-21 samples. The upper left-hand side shows the downregulated proteins. All dots in the center and the bottom are proteins that are not statistically different.

The last volcano plot (Figure 13) shows the comparison of all the mice exposed to chronic stress.

Similar to the volcano plot of APPPS1-21 and WT mice there are not many major differences when APPPS1-21 + CMS and WT + CMS are compared. Only a few of these proteins are related to AD and are otherwise linked to cell physiology, immunology, morphology etc. Of the upregulated proteins related to AD are ApoE, and GFAP, which also were seen in the comparisons of APPPS-21 and WT, as well as APP, which was seen in the comparison of APPPS1-21 + CMS and APPPS1.

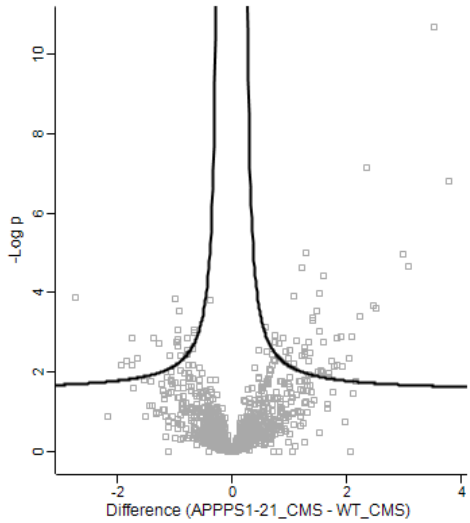


Figure 13: Volcano plot of the differences between APPPS1-21 + CMS and WT + CMS mice. The upper right-hand part of the plot shows all the proteins that are significantly upregulated in the APPPS1-21 + CMS samples compared to the WT + CMS samples. The upper left-hand side shows the downregulated proteins. All dots in the center and the bottom are proteins that are not statistically different.

These volcano plots show clear alterations in the proteome of both the transgenic and WT mice as well as the stressed and non-stress groups. In order to visualize this in another way with additional significance levels Hawaii plots were made prior to PCA and STRING analyses.

Hawaii plot:

Another way to visualize the results from the section above is to make Hawaii plots. This way of plotting data is useful in this project as it is possible to compare the different test groups to a common reference, in this case the healthy WT mice. For the figures the upper left graph is shown the Hawaii plot of APPPS1-21 and WT data, the upper right the APPPS1-21 + CMS and WT data,

and the lower left shows WT + CMS and WT data. The dotted line indicates the 0.05 significance level and the solid line the 0.01 significance level, also called class B and class A respectively. The first graph (Figure 14) shows the Hawaii plots without any highlights of proteins thought to be relevant for this project. In all three graphs there are a significant number of proteins that are statistically significant increased or decreased.

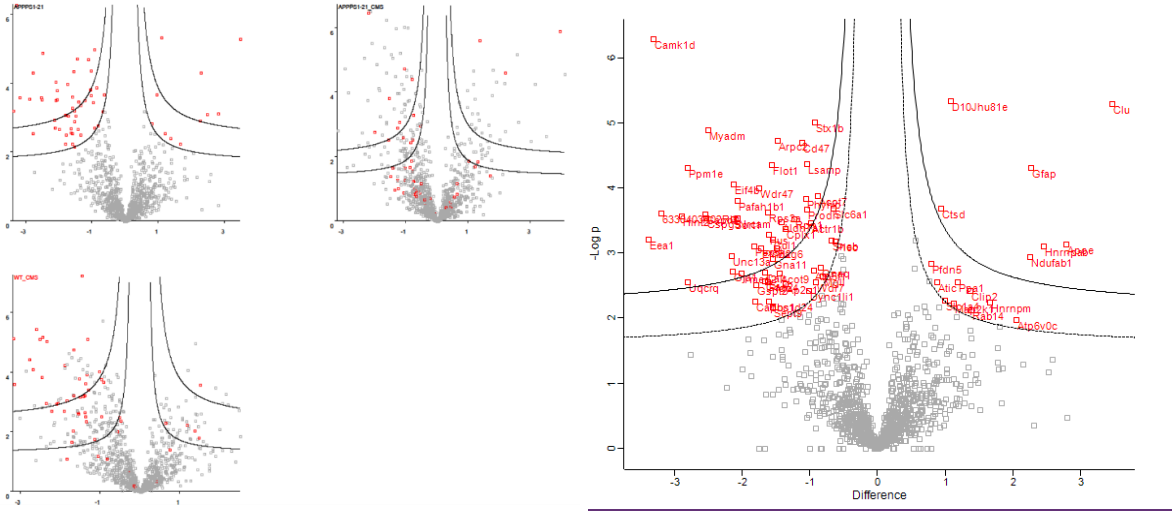


Figure 14: Hawaii plot of the data from label free quantification. The dotted line shows 0.05 significance level and the solid line the 0.01 significance level. (left) All data from the APPPS1-21, APPPS1-21 + CMS, and WT + CMS are compared to the WT data, which is considered a healthy control group. (RIGHT: Significantly regulated proteins of APPPS1-21 vs. WT; gene names highlighted).

Taking a closer look at the proteins, those associated with AD have been highlighted in the next graph (Figure 15). In all three graphs there are proteins above both the 0.05 and 0.01 significance levels, which shows a significant number of proteins involved in AD are being either upregulated or downregulated. This can also be seen in the WT + CMS mice, which indicates a relation between chronic stress and the development of AD. This can also explain why some patients get AD without any family history of AD or any other type of neurodegenerative disorder. Looking at the two transgenic mice groups there are also a higher number of proteins either upregulated or downregulated if they have been exposed to chronic stress, which further supports the theory of the importance of stress for the development of neurodegenerative disorders.

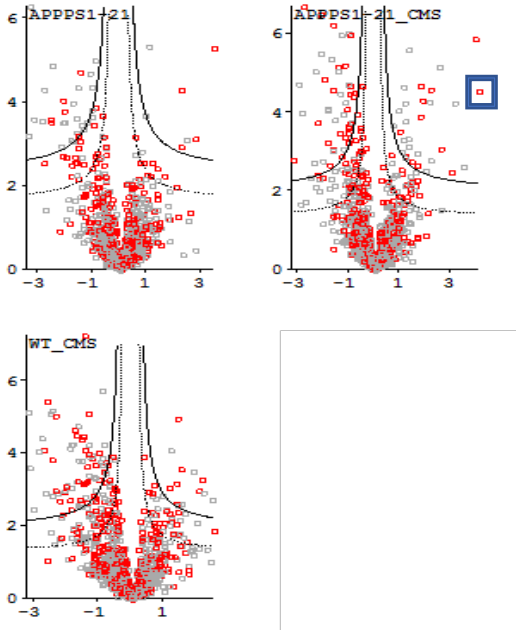


Figure 15: Hawaii plot with highlights of proteins known to be involved in AD. The dotted line shows 0.05 significance level and the solid line the 0.01 significance level. All data from the APPPS1-21, APPPS1-21 + CMS, and WT + CMS are compared to the WT data, which is considered a healthy control group. The blue box shows the placement of APP, which is only upregulated in APPS1-21 + CMS.

Because this project is investigating the relation between AD and stress, another Hawaii plot was made with the highlights of proteins involved in stress, which covers different types of cellular stress, e.g. oxidative stress.

Looking at the graphs (Figure 16) the proteins involved in stress are highlighted. A high number of proteins involved in stress have been identified in all samples, but only a minor part of them are either statistically significantly increased or decreased compared to the WT mice.

In the APPPS1-21 group there are few proteins involved in stress that have been either upregulated or downregulated, even though these mice were not exposed to chronic mild stress.

As expected both test groups exposed to chronic stress have a higher number of statistically significant results than in the WT group. Comparing the two stressed groups, there are some minor differences in the number of proteins that are upregulated or downregulated as well as what significance level the change is at. In the WT + CMS groups there is a lower number of proteins that are increased when compared to APPPS1-21 + CMS, especially at the 0.01 significance level, but looking at the downregulated proteins there are not any major differences between these test groups.

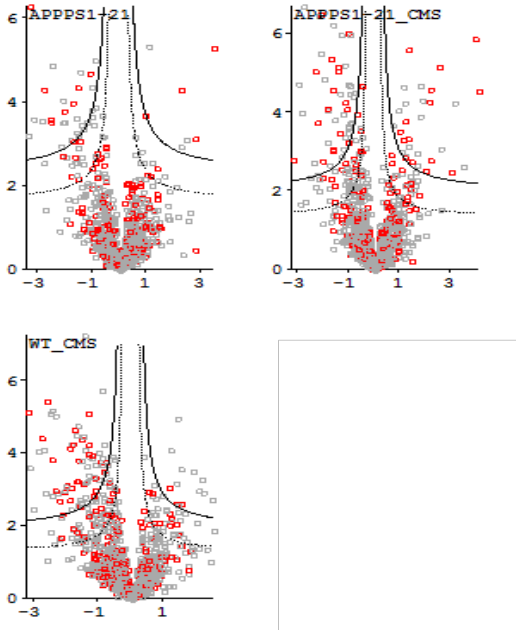


Figure 16: Hawaii plot with highlights of proteins known to be involved in stress. The dotted line shows 0.05 significance level and the solid line the 0.01 significance level. All data from the APPPS1-21, APPPS1-21 + CMS, and WT + CMS are compared to the WT data, which is considered a healthy control group.

Because the tau protein is involved in the pathogenesis of AD it was searched for in the Hawaii plots, which is shown in Figure 17. In both APPPS1-21 and APPPS1-21 + CMS the tau protein is detected, but there is no statistically significant difference between them and WT. In the WT + CMS the protein has not been detected.

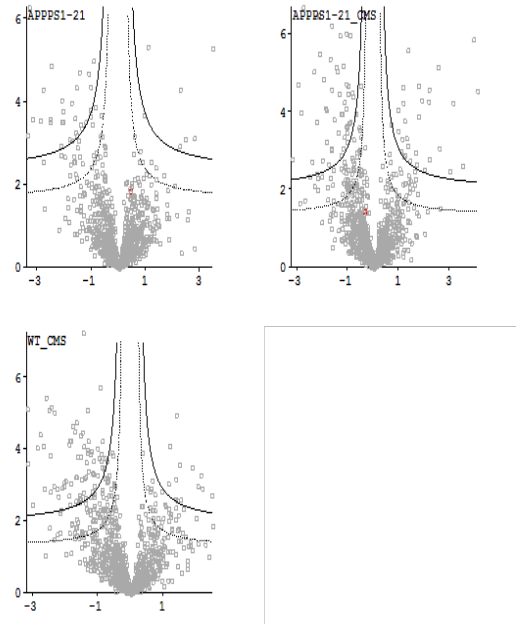


Figure 17: Hawaii plot with highlights of the tau protein. The dotted line shows 0.05 significance level and the solid line the 0.01 significance level. All data from the APPPS1-21, APPPS1-21 + CMS, and WT + CMS are compared to the WT data, which is considered a healthy control group.

A part of the experimental setup was to create sleep disturbances in the mice by creating the 10/10 light/dark cycles. The initial behavioral studies showed the activity of the mice in the two phases changed after being exposed to the change in light and dark phases, and literature also suggests sleep deprivation as a factor in AD. A Hawaii plot was therefore made with highlights of all proteins known to be involved with the circadian cycle (Figure 18).

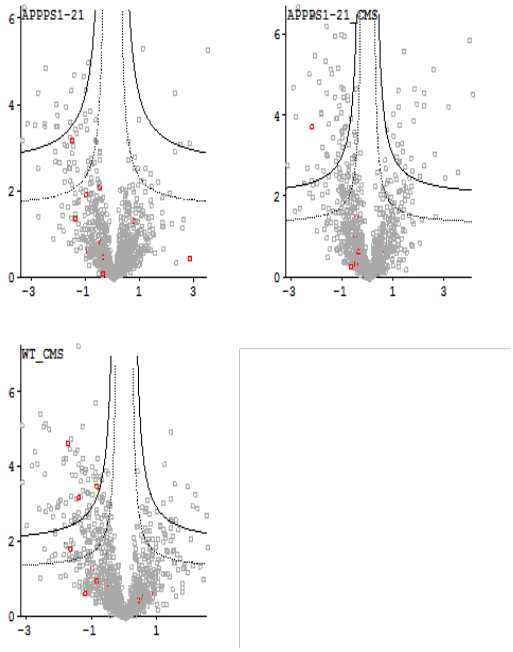


Figure 18: Hawaii plot with highlights of proteins involved with the circadian cycle. The dotted line shows 0.05 significance level and the solid line the 0.01 significance level. All data from the APPPS1-21, APPPS1-21 + CMS, and WT + CMS are compared to the WT data, which is considered a healthy control group.

In all three graphs are proteins statistically significantly decreased compared to the WT mice. I the WT + CMS has been identified three proteins with a p-value < 0.01 and one protein with a p-value < 0.05. In the APPPS1-21 is one protein with a p-value < 0.05 and the APPPS1-21 + CMS is one with a p-value < 0.01.

These findings might be crucial for the explanation of the results of the behavioral studies conducted in the PhD project prior to these experiments. As the circadian cycle normally is very closely controlled by feedback mechanisms, the decrease in the proteins might impair those feedback mechanisms. By impairing this cycle the ability to distinguish between the normal light/dark phases of a day would disappear, thereby explaining the altered activity levels of the mice during the light phase where they would normally be asleep.

Principal component analysis:

In order to investigate correlations between different variables in the data set from the label free quantification a PCA was performed. Below are shown three plots (Figure 19-21) of the first four components in pairs. The first figure shows components 1 and 2, the second 2 and 3, and the last 3 and 4, and thereby showing the variance from greatest to smallest.
The different test groups are represented on the figures as WT (orange square), APPPS1-21 (blue square), WT + CMS (brown plus), and APPPS1-21 + CMS (green plus).

The first graph (Figure 19) shows components 1 and 2, which accounts for the greatest variance in the test samples, 17 % and 10.5 % respectively. The purpose of performing a PCA is to look for variances that are similar in groups and thereby grouping them together. In this project there are no clear cut grouping of the samples in component 1 and 2, though it appears as a slight division of layers with WT in the bottom and the three other groups mixed together.

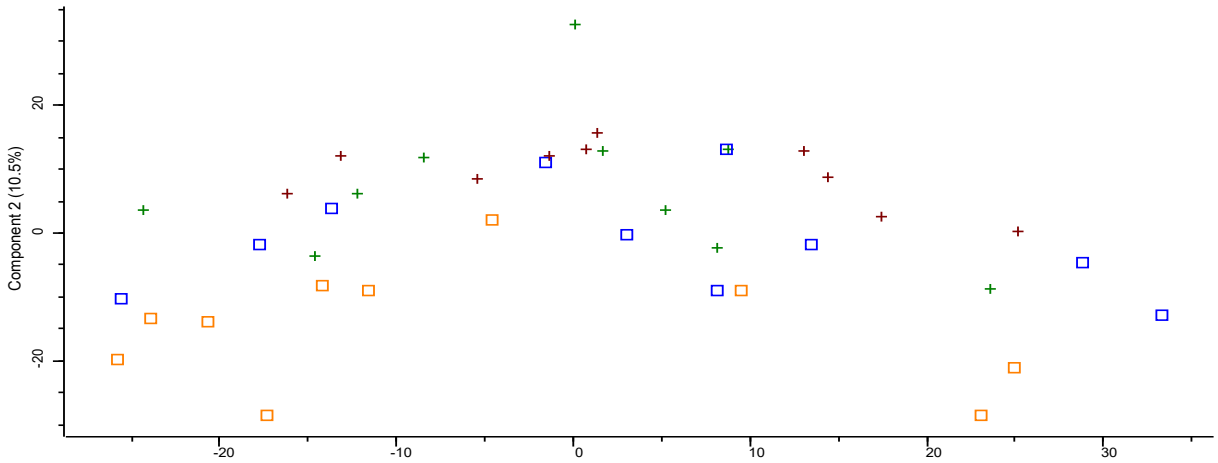


Figure 19: Principal Component analysis after label free quantification of hippocampus samples. The X axis show component 1 and the Y axis shows component 2. Orange: WT, blue: APPPS1-21, brown: WT + CMS, green: APPPS1-21 + CMS. First component accounts for 17 % of the variance and component 2 10.5 %. There is no clear grouping, but a vague layer division with the WT group at the bottom.

As component 1 and 2 did not show a clear grouping of the test groups component 2 and 3 were tested (see Figure 20). Unlike component 1 and 2 the graph with component 2 and 3 shows a clear grouping with a minor overlap in the center. The grouping are WT on the left, APPPS1-21 in the bottom, WT + CMS on the right side, and APPPS1-21 + CMS in the top.

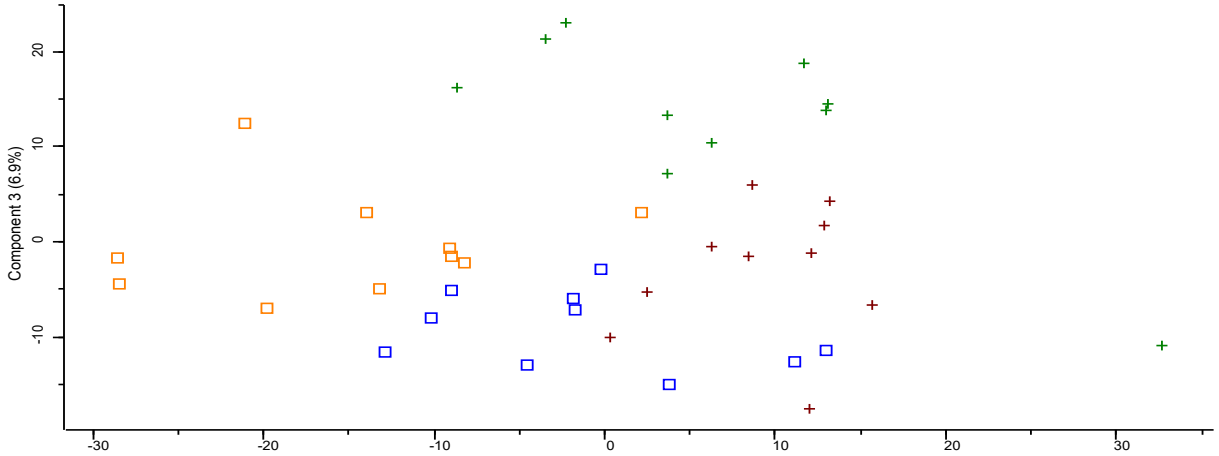


Figure 20: Principal Component analysis after label free quantification of hippocampus samples. The X axis show component 2 and the Y axis shows component 3. Orange: WT, blue: APPPS1-21, brown: WT + CMS, green: APPPS1-21 + CMS. Component 2 accounts for 10.5 % of the variance and component 3 6.9 %.Unlike component 1 and 2 there is a clear grouping of the samples with WT to the left, APPPS1-21 at the bottom, WT + CMS to the right, and APPPS1-21 in the top.

In order to investigate if there is a more distinct grouping than the case with component 2 and 3 a graph with component 3 and 4 was made (see Figure 21).

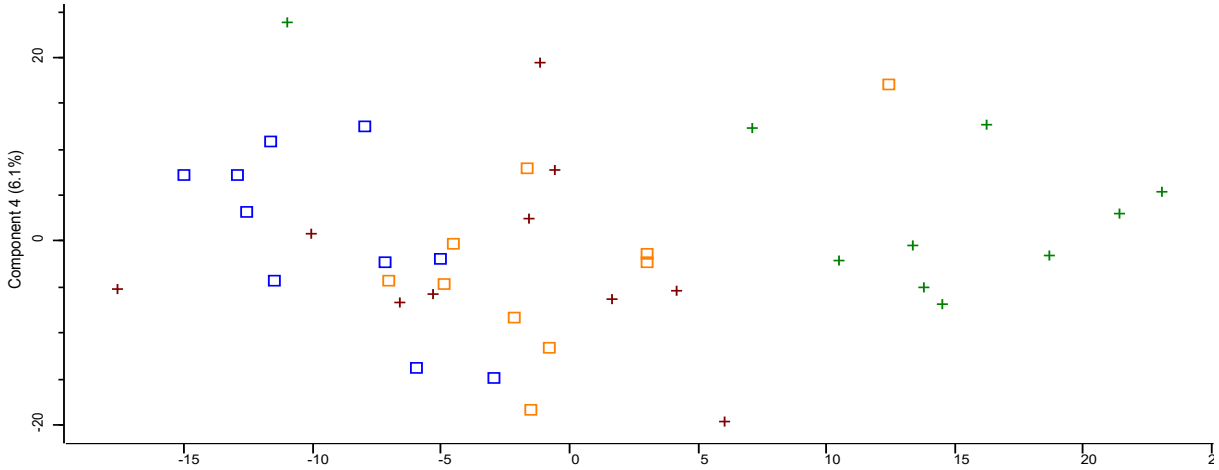


Figure 21: Principal Component analysis after label free quantification of hippocampus samples. The X axis show component 3 and the Y axis shows component 4. Orange: WT, blue: APPPS1-21, brown: WT + CMS, green: APPPS1-21 + CMS

The grouping of the data with component 3 and 4 is not as clear as with component 2 and 3, but the way of the grouping might be important for AD. The WT and WT + CMS samples are grouping together and APPPS1-21 is place to the left of it and APPPS1-21 + CMS to the right.

STRING analysis:

In order to investigate the relation between the factors resulting in the groupings in the PCAs STRING analyses were performed.

The first STRING analysis was performed to investigate what factors were different in the four test groups when component 2 and 3 were plotted (see Figure 20) (see Appendix B for figures).
Looking at the related function of the proteins involved with the placement of the WT group. Using the output from both the label free quantification and the STRING analysis shows the proteins are involved in binding of proteins and the normal function of cells, e.g. metabolism and signaling. The analysis also showed that many of the proteins involved were posttranslational modified, including acetylation and phosphorylation.
The APPS1-21 results also showed proteins involved in the normal cell function as with the WT group, but there were also proteins associated with ageing, behavior, and AD. Phosphorylation, acetylation, and methylations were also found. Further investigation of the methylated proteins showed they are, among other processes, involved in apoptosis, cell growth, dopaminergic neurons, and binding of proteins.
Just with the WT and APPPS1-21 groups proteins of importance also included proteins involved in the cellular function and protein binding. Besides these proteins the WT + CMS group also showed proteins involved in binding of enzymes and development of the nervous system, including guidance of the axons. Phosphorylation, acetylation, proteins involved in AD, and the metabolism were also found.
The APPPS1-21 + CMS group found proteins involved in protein binding, cellular functions, catalytic properties, and responses to nitrogen-containing compounds. Chaperones were also found, and the patterns of phosphorylation's and acetylation's shows that the chaperones all were modified with at least one of these modifications. AD related proteins were also found and a few of them were also phosphorylated.
In order to investigate other factors that have an influence on the groupings another STRING analysis was performed with the results from the plot of component 3 and 4 (see Figure 21), as the grouping is different compared to component 2 and 3 (see pictures in Appendix C).
First looking at the plot it is clear that WT and WT + CMS are grouping in the same area, which also means the proteins placing them here are the same in the two groups, and according to the data some of these proteins are linked to normal cellular functions, protein binding, transport as well as phosphorylated and acetylated proteins. A surprising results is though that some of the proteins identified in this area is linked to both AD, Parkinson's disease, and Huntington's disease, which all are neurodegenerative disorders. Just like the STRING analysis of WT + CMS in component 2 and

3, these proteins have been identified, which indicates the stress of the WT mice might have a crucial role in the development of AD and other types of neurodegenerative disorders.
The difference in APPPS1-21 and WT are not major, but methylations can be detected in the transgenic group, and not the others. The proteins associated with AD, Parkinson's disease, dopaminergic synapses etc. were also identified in this analysis.
Like with the APPPS1-21 group APPPS1-21 + CMS also shows the same proteins as being significant with component 3 and 4 as in component 2 and 3, which indicates other factors are important for the groupings.
Similar the significantly regulated proteins comparing the APPPS1-21 + CMS to the WT group also shows distinct regulated clusters related to AD, Parkinson's disease, dopaminergic synapses (Appendix B).

Detailed analysis for post-translational modifications:

Datamining by BSI PEAKS de novo based searching of MS data was used in order to search for the amount of PTMs present in the hippocampus samples as literature suggests the pattern of PTMs are altered in AD. In table 2 is shown a part of the table from the PEAKS search (see Appendix D for full list) of PTMs.

Table 2: Post translational modifications suspected to be relevant for the pathogenesis of Alzheimer's Disease.
#PSM: number of Peptide-Spectrum Match. -10lgP: -10·Log10(p-value). AScore: confidence of the site of the modification.
S: Serine, T: Threonine, Y: Tyrosine, H: Histidine, K: Lysine.

Name	ΔMass	Position	#PSM	-10lgP	Abundance	AScore
Phosphorylation	79.97	STY	1533	128.34	8.39E7	61.3
HNE	146.12	HK	95	56.59	9.26E8	1000
Nitro	44.99	Y	5	35.33	1.12E7	77.79

The phosphorylation is the PTM of the chosen with the highest discovery rate (1533 hits), but the Ascore is relatively low compared to the two others, which means there is a relatively higher risk of false identification sites. The -10lgP is also high, which reflects a high p-value.
The 4-hydroxynoneal (HNE) modification has only 95 hits, but according to the -10lgP and AScore the identifications made are quite exact. Similar is the nitro modification (5 hits) which also have a low -10lgP, but the AScore is also low, which means the false identification risk is relatively high.

Discussion

Literature on AD and neurodegeneration in general have implicated some proteins as being crucial for the development and severity of the disease, e.g. ApoE[4,11,59–61], GFAP that indicates astrocyte activation associated with neurodegeneration[21,46–49], and APP because it is the precursor for β-amyloid.

Taking a closer look at ApoE, the literature suggests the isoform of ApoE is crucial for its function[20] with ApoE2 being more protective in relation to AD and ApoE4 increasing the risk of AD[4,11,59–61]. At the volcano plots there are found statistically significant upregulations of ApoE in the comparisons of APPPS1-21 – WT and APPPS1-21 + CMS – WT + CMS, and downregulations in the comparisons of WT + CMS – WT and APPPS1-21 + CMS – APPPS1-21. These results suggests the mutations linked to AD increases the total amount of ApoE in hippocampus, but chronic stress reduces the amount. Though the transgenic mice have a higher level of ApoE those who were also exposed to chronic stress have a lower level. In order to investigate the level of ApoE in the APPPS1-21 + CMS group compared to the healthy controls another volcano plot was made (Figure 22).

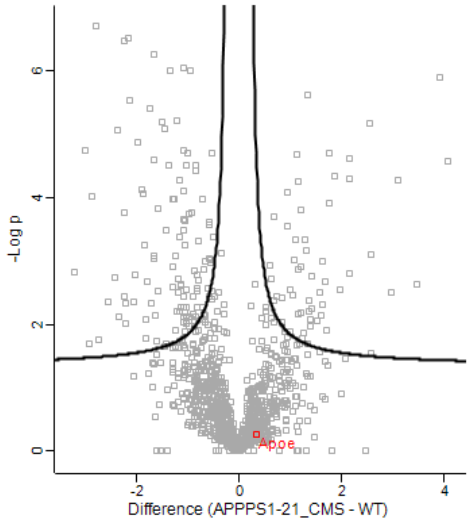


Figure 22: Volcano plot of the differences between APPPS1-21 + CMS and WT mice. The upper right-hand part of the plot shows all the proteins that are significantly upregulated in the APPPS1-21 + CMS samples compared to the WT + CMS samples. The upper left-hand side shows the downregulated proteins. All dots in the center and the bottom are proteins that are not statistically different. ApoE is marked with red.

The volcano plot shows that there are no statistically significant differences in the levels of ApoE in the APPPS1-21 + CMS and WT groups. This can suggest that, if the upregulation in the APPPS1-21 group is a try to protect the brain from accumulation of β-amyloid, the stress might prevent this and worsen the accumulation. This theory is also supported by the literature on stress and AD, that

has shown that stress increases the risk of developing AD[3,12,16,17,27–33,35]. This result might point towards a biological connection between the stress and the AD risk.
APP is another protein crucial for development of AD due to the faulty cleavage leading to the production of β-amyloid. In the volcano plot of APPPS1-21 + CMS – APPPS1-21 and APPPS1-21 + CMS – WT + CMS there were a statistically significant upregulation of the APP levels, but no changes were found in the plots of WT + CMS – WT and APPPS1-21 – WT. These results suggest the mutation of the risk associated genes are not enough for the mice to have an increased APP expression, but other factors are needed in order to develop AD. This also supports the role of stress to develop AD as also seen with ApoE in the section above, and it can also explain why the upregulation of APP is only seen in the APPPS1-21 + CMS group regardless of the group it is compared to.
A protein that is not directly linked to AD itself, but to the astrocytes that are thought to have a crucial role in the homeostasis in the brain is GFAP. The volcano plots show a significant upregulation of GFAP in the transgenic mice, but not in WT mice independent of the stress exposure. An upregulation is seen in the comparison of the APPPS1-21 and WT mice but not in the comparison of APPPS1-21 + CMS and APPPS1-21, which indicates the upregulation is based on the presence of the mutated genes and not the stress. The literature on astrocytes tell that when the astrocytes are activated, and thereby also hypertrophic, the expression of GFAP is increased, and studies have concluded this happens in the proximity to β-amyloid[46–49].

Investigating the PCA grouping of the test samples shown in Figure 20 and 21, with component 2 and 3 respectively component 3 and 4, the way of the grouping might be very important for explaining the connections between AD, chronic stress, and sleeping patterns.
In Figure 23 below is shown a modification of Figure 20. The figure shows the effect of chronic stress on the APPPS1-21 and WT mice as indicated by the blue arrows. The stress groups are parallel displaced compared to the non-stressed, which indicates the effect of chronic stress is the same independent of the genetic background. A STRING analysis showed that the proteins responsible for the parallel displacement of the stress mice includes proteins related to AD, neuronal growth and projections, ageing, behavior, and binding of proteins.

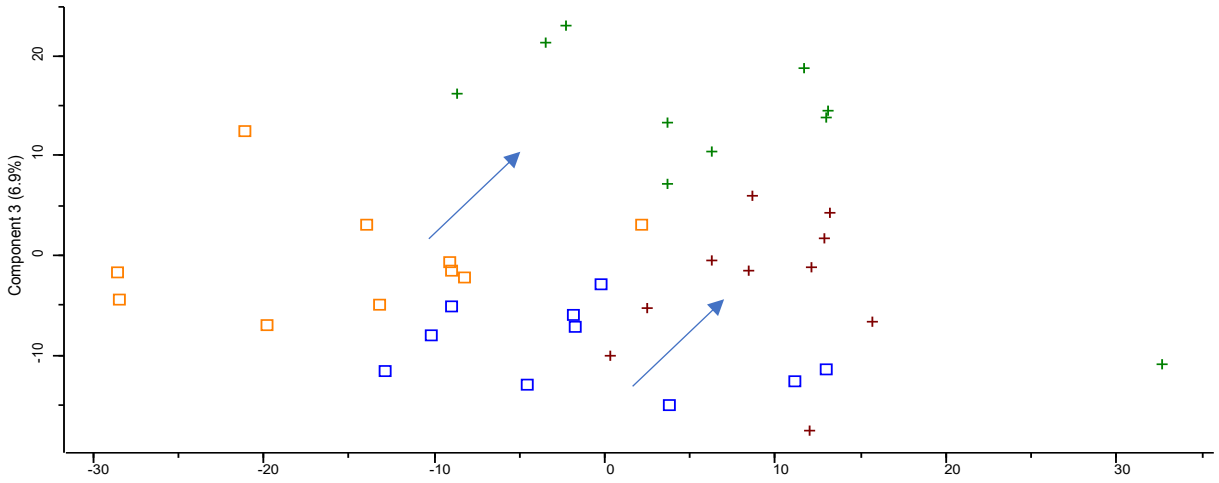


Figure 23: Modification of Figure 14. The arrows indicate the effect of the chronic stress on the APPS1-21 and WT groups respectively. Orange: WT, blue: APPPS1-21, brown: WT + CMS, green: APPPS1-21 + CMS.

The grouping of the samples in Figure 21 there is an interesting pattern. WT and WT + CMS are both placed in the middle of the graph with APPPS1-21 to the left of it and APPPS1-21 + CMS to the right. As with component 2 and 3 the exact reasons for the grouping is hard to find, but it makes WT and WT + CMS group together. APPPS1-21 is places to the left, and the proteins involved with this placement as well as the placement of APPPS1-21 + CMS to the right has been identified as being involved with e.g. AD, Parkinson's disease, and dopaminergic synapses. But the lack of grouping of the transgenic mice like with the WT might be due to the detection of methylations in the APPPS1-21 group. Another reason for the placement of the transgenic mice could be due to differences in the abundance of different proteins, with APPPS1-21 containing less than WT and WT + CMS and APPPS1-21 + CMS containing more.

Very important for this project is to investigate the effect of stress, especially on subjects with AD. Looking into the placement of the proteins related to stress in the scatter plot used to identify grouping-related proteins surprisingly shows that no proteins identified as being involved in the grouping of the mice have been linked to stress and cellular responses to stress. In Figure 24 is shown the scatter plots for component 2 and 3 to the right and component 3 and 4 to the left. Similarly there is no pattern in component 1 and 2 (figure not shown). As seen all the identified proteins are equally distributed over most of the plot and not confined to one area as would have been expected from the PCA plot of component 2 and 3, where a clear line between stressed and non-stressed mice can be seen.

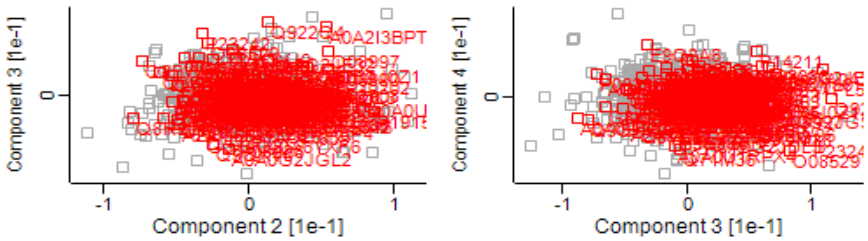


Figure 24: Scatter plots of proteins for the PCA analysis of component 2 and 3 respectively component 3 and 4. All proteins associated with stress or cellular responses to stress are marked with red. There are no pattern in the placement of these proteins, which also means they are not important for the grouping seen in the PCA plots.

So even though literature[3,12,16,17,27–33,35] indicates chronic stress is taking part in the development and severity of AD, it cannot be biologically proven by these analyses, as there are no clear grouping of the proteins involved.

Literature on the brain and how it is clear of waste products have linked poor sleeping and sleep disturbances with both memory problems and neurodegeneration, as well as worse circadian regulation as age increases[13,72–77]. In Figure 25 is shown a volcano plot comparing APPPS1-21 + CMS and WT + CMS as well as APPPS1-21 + CMS and APPPS1-21, both with red highlights of the proteins known to be involved with the circadian cycle. Both plots shows a few statistically significant differences; two upregulations in the comparison of APPPS1-21 + CMS and WT + CMS, as well as one downregulation and two upregulations in the comparison of APPPS1-21 + CMS and APPPS1-21.

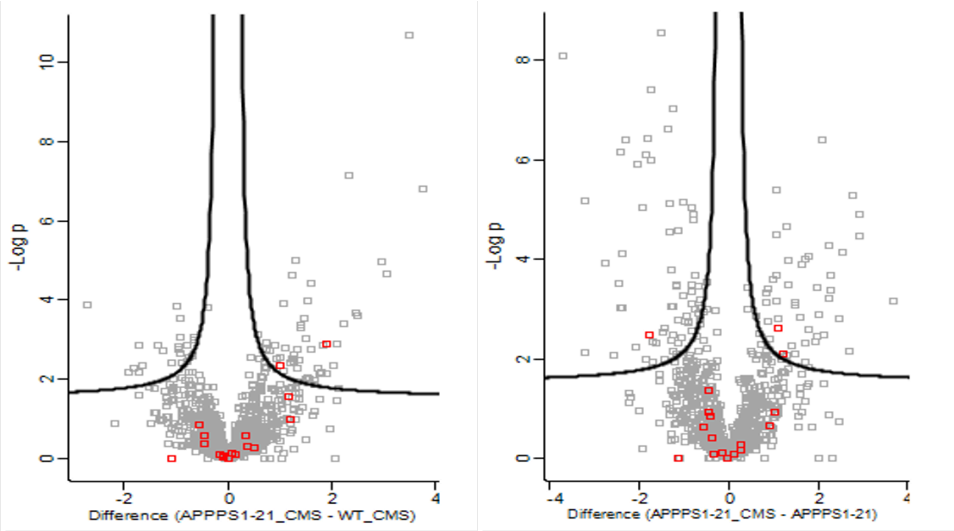


Figure 25: Volcano plot of the differences between APPPS1-21 + CMS and WT + CMS mice (left) and APPPS1-21 + CMS and APPPS1-21 (right). The upper right-hand part of the plot shows all the proteins that are significantly upregulated in the APPPS1-21 + CMS samples compared to the WT + CMS samples. The upper left-hand side shows the downregulated proteins. All dots in the center and the bottom are proteins that are not statistically different. The proteins highlighted with red are associated with the circadian cycle.

These results indicate that though the protein content of the WT + CMS, APPPS1-21, and APPPS1-21 + CMS are lower compared to the WT there are some differences in content between these

groups. These differences could also be seen in the behavioral studies performed prior to these studies. They showed that APPPS1-21 and WT mice had similar activity patterns and appeared as what could be expected from nocturnal animals. The APPPS1-21 + CMS and WT + CMS mice also had similar activity patterns, but there were no statistically significant differences between the light and dark phases in these animals. Below is two scatter plots (Figure 26) showing component 2 and 3 to the left and component 3 and 4 on the right. In both plots the proteins involved with the circadian cycle have been highlighted in red, and shows an interesting pattern.

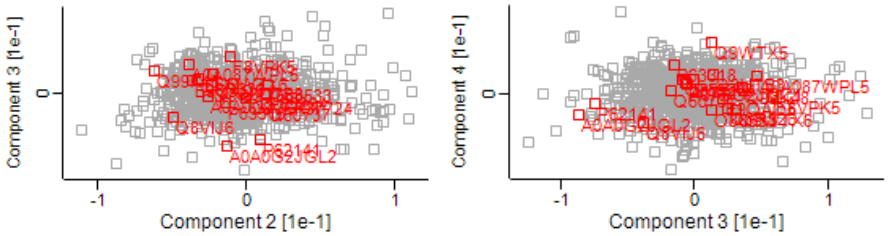


Figure 26: Scatter plots of proteins for the PCA analysis of component 2 and 3 respectively component 3 and 4. All proteins associated with the circadian cycle have been highlighted with red.

The placement of these proteins that have been highlighted coincide with some of the proteins responsible for the grouping of the mice in the PCA plots (Figure 20 and 21). In the plot of component 2 and 3 the proteins involved in the circadian cycle is placed in the areas where the WT, APPPS1-21, and WT + CMS groups are placed on the PCA plot (Figure 20). A similar pattern can be seen with component 3 and 4, where the circadian cycle proteins also line up with the areas where the WT, APPPS1-21, and WT + CMS groups are placed (Figure 21). Interestingly none of the proteins line up with the placement of the APPPS1-21 + CMS group in any of the components. Most clearly shown with component 3 and 4 is that the proteins in the WT and WT + CMS are placed together. The Hawaii plots showed that only four proteins were statistically different in these two groups, which also shows that even though the mice are otherwise healthy there are still some effects of sleep deprivation, which also has been shown in clinical tests[15] where accumulation of β-amyloid was detectable after only one night of sleep deprivation.

The PTM search in PEAKS showed some modifications linked to AD[50,58,59,78–80], but the exact proteins that have been modified are pending further analysis with the data set (data not shown).

Conclusion and perspectivations:

The overall aim of the current study was to substantiate if the murine WT and APPPS1-21 model is applicable for CMS studies using proteomics as technology platform. The performed experiments of this studies gave several interesting results and novel findings providing insight for the research into Alzheimer's disease as many know risk-associated proteins were identified as being statistically significant changed between the four test groups. Overall, the far majority of findings are correlated with known pathways and affected proteins in neurodegenerative diseases. In particular, ApoE, which in human have been associated with the risk of developing familiar Alzheimer's disease (especially ApoE4), was found to be significantly increased in the APPPS1-21 mice, but it was decreased in both APPPS1-21 + CMS and WT + CMS mice, and the APPPS1-21 + CMS was indifferent to the level in the WT mice. The circadian cycle has also been implicated in the progression and severity of AD, e.g. through the purposed glymphatic system. This system purpose that an inadequate drainage and clearance of the brain can lead to an accumulation of different waste products, including β-amyloid. Because this clearance is mainly active during sleep and the results of this project that showed a decrease in some proteins involved in controlling the circadian cycle, this field would be a field interesting for further research.

In a translational and clinical view, the results of this project might be important for further trials. Most interesting is the application of the clear-cut effect of the CMS on the WT group whereby this model may be applicable to other NPD studies and effect studies of behavioral altering drugs (Ayodeji Abdur-Rasheed Asuni; H. Lundbeck A/S, personal communication). The literature and these results points at sleep as an important factor for progression of AD, and therefore it would be interesting to do clinical trials of sleeping in AD or MCI patients, including a randomization of sleeping medications and placebo, in order to investigate if improved sleep can alleviate the symptoms and maybe stop the disease progression over time.

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