UNIVERSITY OF TURKU

MID1 inhibitors to delay disease progression in neurodegenerative diseases

Institute of Biomedicine
MDP in Biomedical Sciences
Drug Discovery and Development
Master's thesis

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Neurodegenerative diseases (NDs) are characterized by neuronal damage where nerve cells degenerate and die. Alzheimer's and Parkinson's diseases are the most prevalent and well-known NDs. Current treatments alleviate the symptoms but are unable to slow the progression of the disease or the destruction of the nerve cells. Therefore, finding novel therapies that could modify the course of neurodegenerative diseases is important.

Midline 1 (MID1), a ubiquitin ligase, is a novel potential target for drug development. The protein forms a complex with protein phosphatase 2A (PP2A) and its subunit α4 and transports them to proteasomes for degradation. MID1-inhibitors aim to prevent the degradation of MID1- α4- PP2Ac complex in proteasomes and thus increase the PP2A protein activity in cells. Through this, tau is dephosphorylated and stabilized in microtubules as PP2A is the main phosphatase to dephosphorylate tau. Tau protein dephosphorylation promotes normal cell metabolism and maintains cell structure of microtubules, preventing cell malfunction and synaptic impairment. This also prevents the formation of the toxic neurofibrillary tangles that damage neurons, and thus the progression of the disease. The purpose of the study was to characterize MID1 as a drug target for small-molecule inhibitors and study how the MID1 modification affects PP2A levels and MID1-α4 -interaction.

The research included MID1 validation and compound testing done at the same time. The experiment started with the validation of MID1 as a drug target. The aim was to silence MID1 from human embryonic kidney 293 cells by using commercial MID1 small interfering RNA (siRNA). Although the results obtained were not statistically significant, the association between MID1 and PP2A is noticeable with the MolPort 6 inhibitor compound.

Key words: Neurodegenerative diseases, MID1-α4-PP2Ac complex, MID1-inhibitors.

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1 Introduction

1.1 Scientific background of neurodegenerative diseases

Neurodegenerative diseases (NDs) are diverse and wide range of sporadic diseases characterized by neuronal damage, impairment and failure of neuronal connections, and progressive loss of nerve cells. The NDs are classified and vary according to different characteristics. These can be clinical features including for example movement disorders or dementia where the symptoms are linked to the damaged areas in the brain. In addition, diseases are also classified according to anatomical distribution which includes for example extrapyramidal disorders, and molecular aberration which is protein based (Kovacs, 2018).

Alzheimer's disease (AD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Creutzfeldt Jakob disease, Lewy body dementia (LBD) and Multiple sclerosis (MS) are examples of serious and significant NDs in central nervous system (CNS), which are characterized by care dependence, as there is currently no curative disease modifying treatments (Agnello and Ciaccio, 2022).

Exact cause and pathophysiology behind the NDs are still unknown, but aging is the biggest risk factor (Hou et al., 2019) Hereditarily rare NDs usually occur in older people. Thus, as the population ages, the incidence and the risk of neurodegenerative diseases increases (Reith, 2018). In other words, the prevalence of age-related NDs, such as AD and PD, become more common world wide since aging seems to be the major risk factor. This is an increasing problem especially in Western countries and in China, where the population is aging rapidly (Congdon and Sigurdsson, 2018; Orf and Walker, 2017).

Factors predisposing to NDs have been found in both genetics and external factors such as environmental factors, but the mechanisms behind disease onset and accurate etiology are still largely unknown and poorly understood (Hou et al., 2019). In neurodegenerative diseases the main pathological causes are aggregation and accumulation of toxic proteins. This aggregation promotes neuronal damage which leads to obstruction of flow of information in the nerve cells. Microtubule-associated protein tau in hyperphosphorylated form, beta-amyloid (Aβ), alpha-synuclein (α-syn) and prion proteins are the most common proteins associated with pathogenesis of NDs which are forming different groups of disorders called e.g., tauopathy, amyloidosis and α-synucleinopathy (Dugger and Dickson, 2017).

So far, molecular epidemiology provides a deeper understanding of disease dynamics, transmission patterns, and pathogen characteristics, which in turn lead to improved disease diagnoses and more effective treatments. As mentioned, deposition and aggregation of toxic proteins such as tau and amyloid-beta in AD or α-syn in PD are the main pathological characteristic features of these neurodegenerative disorders. These proteins damage nerve cells during their aggregation process. So, NDs are multifaceted and involve intricate interactions between various cell types, genetic factors, and environmental influences (Bertram, 2005).

In addition to accumulation of abnormal proteins, several mechanisms that damage and probably kill neurons during neurodegeneration have been identified in preclinical studies. Prolonged inflammatory reaction, mitochondrial dysfunction, and oxidative stress are examples of detrimental mechanisms targeting the nervous system. They trigger apoptosis and lead to decreased autophagy where their dysregulation seems to be implicated in various NDs. Increasing autophagy is essential for clearing misfolded proteins and damaged organelles since accumulation of abnormal proteins and impaired clearance mechanisms contribute to the onset and progression of NDs. (Erekat, 2022; Kurtishi et al., 2019).

Briefly summarized, in NDs, various toxic proteins are one of the main pathological causes. Aggregation and accumulation of toxic proteins in neurons such as α-syn in PD and hyperphosphorylated tau in AD, result in inhibition of information flow at synapses and reduce reversible autophagy, contributing to neuronal cell damage. As mentioned, the process of damaging nerve cells during neuropathy eventually leads the nerve cells to degenerate and die progressively. Furthermore, neurofibrillary tangles (NFTs) in AD, Lewy bodies in PD and amyloid deposits, are observed which are hallmarks of diseases (Dugger and Dickson, 2017).

Additionally, the gene mutations underlying neuronal damage are important to identify since they adversely affect many disease's factors such as aberrant processing and accumulation of the misfolded protein in the neuron or mitochondrial insufficiency. Together, these lead to damage and death of the nerve cells and loss of dopaminergic neurons (Macdonald et al., 2018; Taylor et al., 2002). Figure 1 summarizes these common risk factors and pathological processes of NDs.

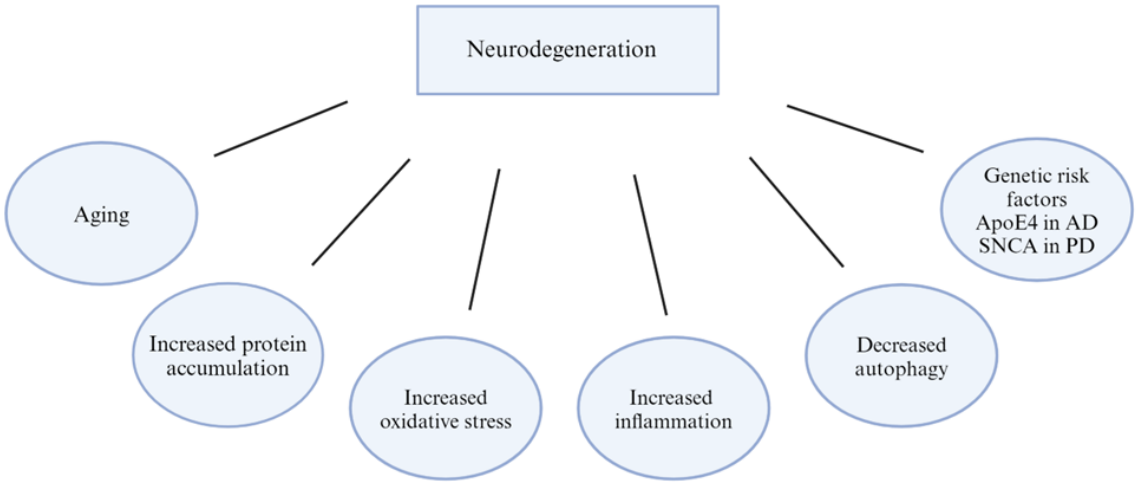

Figure 1. The most common risk factors and pathological processes of NDs. AD = Alzheimer's disease, PD = Parkinson's disease, ApoE4 = Apolipoprotein E 4 allele, SNCA = Gene in PD encodes α-synuclein, NDs = Neurodegenerative diseases. Created by BioRender.

1.2 Alzheimer's disease

AD, which was first described by Alois Alzheimer in 1901 in a female patient, is progressive and the most common chronic neurodegenerative disorder that affects cognitive functions, memory, and behaviour. AD is the cause of progressive memory symptoms and dementia in at least 70% of cases. The disease is characterized by memory loss and is significantly the most common reason for developing dementia leading eventually to the inability to recognize loved ones and survive the simplest of daily tasks. Typical neuropathological findings of the disease include extracellular neuritis plaques consisting of Aβ and intracellular NFTs consisting of abnormally hyperphosphorylated tau protein (Jellinger et al., 1990; O'Brien and Wong, 2011).

1.2.1 The stages of AD

AD proceeds slowly, individually and symptoms vary between people. It can be typically classified into different clinical stages. Pre-clinical AD can start asymptomatically for 20 years with changes in the brain without diagnosis (Crous-Bou et al., 2017). The disease is commonly diagnosed during the mild-early stage when learning and memory function are impaired. At this stage, symptoms include forgetting the names of things and objects, confusion in unfamiliar surroundings and difficulties in balance and speaking. During moderate-middle stage, symptoms deteriorate progressively. The need for support increases in addition to aggressive or irritable behavioural disorders and long-term memory impairment. At severe-late AD, patients are completely dependent on caregivers and speech production is limited or inhibited. Decreased muscle mass and mobility

will eventually lead to bedridden life and the disease is eventually fatal (Breijyeh and Karaman, 2020).

1.2.2 Prevalence and incidence of AD

AD causes up to 60 – 80 percent of all diagnosed dementia cases. Dementia is more common in women, and it is estimated that around 100 million people will suffer from dementia by 2050. It is also increasing and one of the significant healthcare problems of the 21st century (Bertram et al., 2007; Crous-Bou et al., 2017; Nichols et al., 2019).

The incidence of a destructive and dementing AD increases with age which is the biggest and primary risk factor for the disease. AD occurs mainly in elderly people, over 65 years of age (Hou et al., 2019). One paper published in 2021 reported that in the United States 6.2 million people over the age of 65 have been diagnosed with Alzheimer. This means approximately the prevalence of 10%. It is estimated that the incidence of AD will grow, and it is expected to rise to 13.8 million by the end of 2050 in US (Orf and Walker, 2017; Parums, 2021; Soria Lopez et al., 2019).

1.2.3 Etiology of AD

The complex AD has several gene mutations as risk factors located in chromosomes 1, 14, 19 and 21. Mutated genes included in these chromosomes are apolipoprotein E 4 allele (ApoE4), presenilin-1 (PSEN1), presenilin-2 (PSEN2) and amyloid precursor protein (APP) (Lanoiselée et al., 2017).

ApoE4, identified in chromosome 19, is the largest genetic risk factor along with aging for multifactorial and most common late-onset AD form. The gene predisposes to the disease in more than 40 % of cases. The allele ε4 is more important in removing Aβ protein in the brain than gene isoforms ε2 and ε3. This probably explains why ε4 is the most significant of these than other isoforms. It has also been estimated that ε2 isoform would decrease the risk of disease (Selkoe and Hardy, 2016; Strittmatter et al., 1993).

APP has been identified in chromosome 21. Although most cases of AD are sporadic, mutations and changes in APP are found especially behind the AD's uncommon familial early onset form where the disease starts at the age of under 65 years. In addition to APP, PSEN1 found in chromosome 14 and PSEN2 found in chromosome 1 are commonly associated with this early onset form of the disease. What all of these have in common, is their unfavorable effect on Aβ production and thus leading to increased levels of $A\beta_{42}$ that lead to neurodegeneration (Elbaz et al., 2007; Lane

et al., 2018; Lanoiselée et al., 2017). The information and meanings of the genes are summarized in Table 1.

Even though mutations explain only a small part of the mechanisms behind the disease, it is important to recognize the molecular significance of disease-related mutations as mutations have made it possible to find proteins that are relevant to the disease. These changes in protein function can affect the onset or course of the disease. Therefore, it is significant to understand better the structures of molecules and the changes that take place in them, as well as the functions of molecules and their interactions with other molecules as many changes occur in enzymes during development of NDs (Balestrino & Schapira, 2020). An example of this is the mutation in APP that affects both cleavage and aggregation of Aβ precursor peptide or impairment of acetylcholine (ACh) function in AD, where the activity of its synthesizing choline acetyltransferase is reduced. (Scheltens et al., 2016). APP mutations increase β-secretase cleavage and PSEN1 / PSEN2 mutations increase γ-secretase activity (Figure 4) that are behind the formation of toxic amyloid plaque, a hallmark of AD.

So, even if an individual has inherited the mutated apolipoprotein gene or other genes, it does not unequivocally determine the onset of the disease. Mutations cover only about 10 percent of all AD cases. Mainly they are age related and sporadic cases with a late onset (Tilley et al., 1998).

Table 1. A summary of the most common and predisposing genes in Alzheimer's disease

AD = Alzheimer's disease, APOE = Apolipoprotein E, PSEN1 = presenilin 1, PSEN2 = presenilin 2, APP = amyloid precursor protein, Aβ = beta-amyloid.
(Adapted from Lane et al., 2018; Selkoe and Hardy, 2016; Tilley et al., 1998).

Gene	Chromosomal location	Estimated disease cases	Other information
APOE	19q13.2	> 40 %	Mainly late-onset AD but also early onset AD Familial / Sporadic Risk factor ε4 Other alleles: ε2 and ε3 Glycoprotein: 34 kDa
PSEN1	14q24	Up to 80 %	Familial early-onset AD Transmembrane protein Involved in increased Aβ production
PSEN2	1q31-42	~ 20 %	Familial early-onset AD Involved in increased Aβ production Number of mutations: 2
APP	21q21.1	Up to 5 %	Familial early-onset AD Changes on APP mutations: → increased Aβ levels

1.2.4 Pathophysiology of AD

The main cause of AD is still unknown. Important and remarkable biomarkers are cerebrospinal fluid derived specific proteins. Increased levels of APP are one of these CSF-related proteins. It affects the presence of aggregation-prone amyloid-beta peptides (Aβ1-40 and Aβ1-42), leading to the formation of toxic amyloid and senile plaques in the brain. Also, increased levels of total tau are a useful biomarker that reflects the intensity of the disease. Especially if phosphorylated forms of tau-18 and tau-217 are present. They are responsible for another pathological hallmark called neurofibrillary tangles and correlate with pathological changes of the disease (Apostolova et al., 2010; Grøntvedt et al., 2018; Horie et al., 2021).

The complex disease varies between clinical and genetical aspects. It has also variation in protein accumulations meaning e.g., amyloid composition and differences in distribution of both tau and amyloid (Ferrari and Sorbi, 2021). Tau pathology and the amyloid cascade hypothesis have long been the main pathological factors. However, studies have found several mechanisms that damage and probably kill neurons during neurodegeneration. Nowadays, it is believed that, along with damage of protein processing, the oxidative stress, prolonged inflammatory reaction, mitochondrial dysfunction, and vascular damages are all related to the pathological onset of the disease. Protein aggregation may trigger these above mentioned pathological mechanisms, which in turn also lead to the breakdown of proteins and thus negatively affect the onset of the disease. These pathological changes have been observed in the brain of AD. These are not unequivocal pathological factors. Many of them are related to each other and thus trigger the disease process. (Chiti and Dobson, 2017; Menzies et al., 2017). For example, a disturbance in the metabolism of amyloid-beta peptide affects its accumulation in both extracellular senile and amyloid plaques and blood vessel walls. Also, activated microglia together with senile plaques could lead to releasing of glutamate in synaptic areas, which in turn leads to local excitotoxicity in neuronal synapses and thus contributes to neuronal degeneration and dysfunction (Piani et al., 1992). The figure 2 summarizes the most important pathological hypotheses and pathologies of the disease and the associated neurodegenerative characteristics and mechanisms.

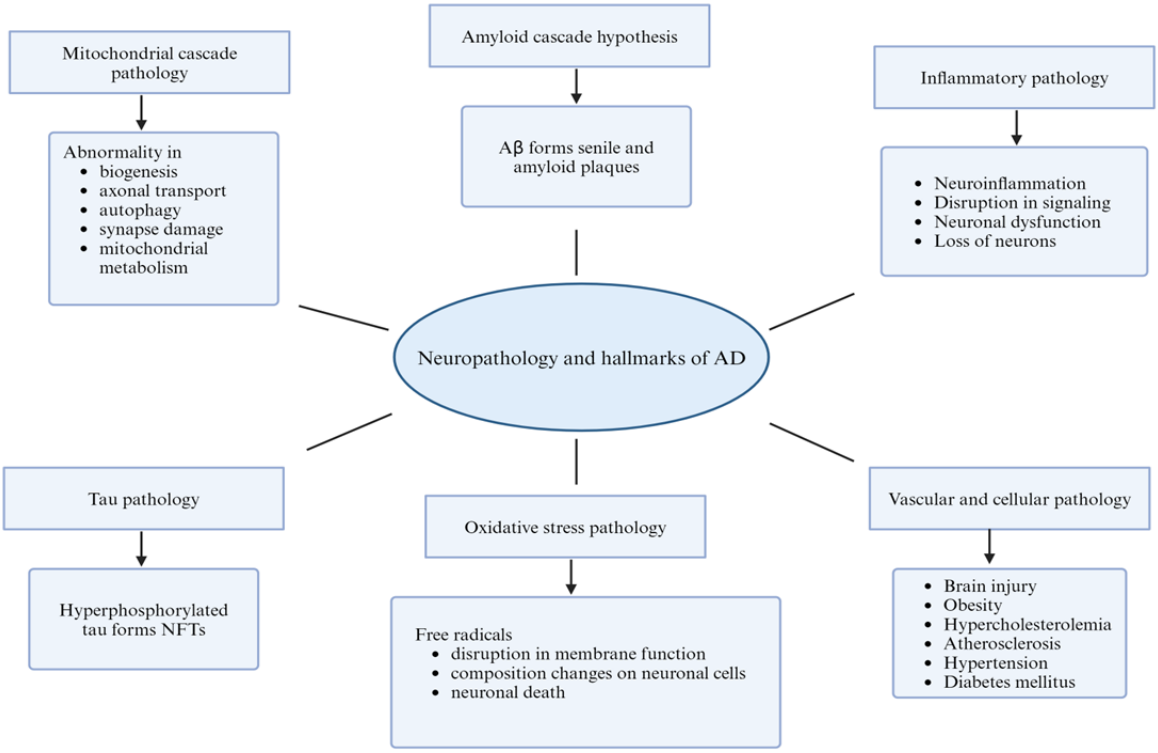

Figure 2. The mechanisms of AD are still largely unknown. Multifactorial AD is highly aging-related. The development of the disease is influenced by many different stress factors that affect the brain. The disease is not caused by a single cause but is the result of the combined effect of many different factors.
AD = Alzheimer's disease. Created by BioRender.

AD can start asymptomatically decades earlier with pathological brain changes before diagnosis. At this point, toxic and abnormal protein deposits such as amyloid plaques and neurofibrillary tangles begin to produce changes in the brain that lead to the disconnection and death of healthy cells.

Pathological changes and neuronal death explain the shrinkage of the brain and the symptoms of the disease. As shown in Figure 3, AD is characterized by the progressive loss and damage of neurons and synapses which manifests as a thinner cerebral cortex and shrinkage in hippocampus, which are the brain regions associated with memory and learning. As the disease progresses, the brain undergoes widespread damage which becomes increasingly severe over time, causing more extensive damage and further deterioration of cognitive functions. This neuronal and synaptic loss with plaques and NFTs is a hallmark pathological features of the disease and is closely associated with the cognitive and functional decline seen in affected individuals. These pathological hallmarks can be observed from AD patient's postmortem samples (Plum, 1986).

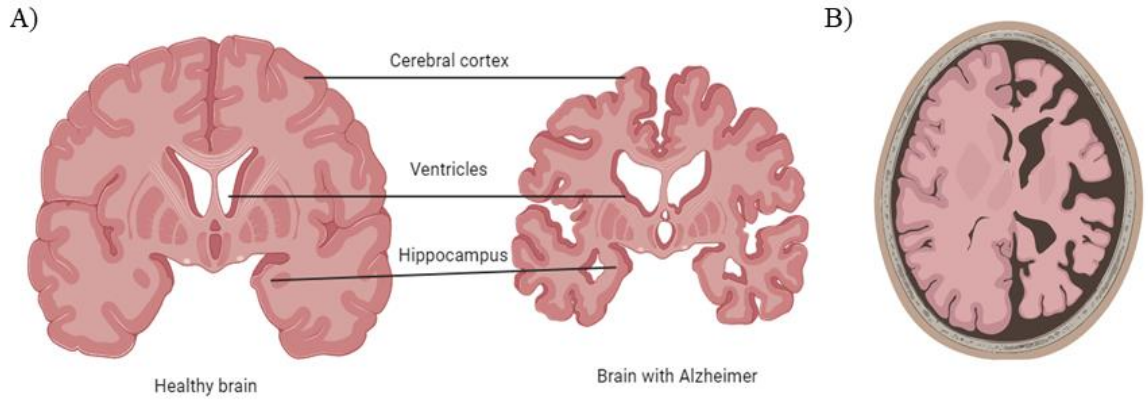

Figure 3. A) Transverse section of the pathophysiological brain changes in AD. A normal healthy brain is shown on the left and a brain with AD on the right. Pathological brain changes start in the neocortex and hippocampus. Nerve cells are destroyed especially in the nucleus basalis, where the function of the biochemically important neurotransmitter, ACh, decreases. Neurons are destroyed from the base of the forebrain up to the cerebral cortex. Characteristics appears as enlarged ventricles and shrinkage of both cerebral cortex and hippocampus. B) Pathologic plaques and NFTs explain morphological brain changes. In the AD brain, they occur in the grey area and spread both in the neocortex and paleocortex. A brain atrophy manifest as shrinkage and a reduction in the brain tissue mass.
AD = Alzheimer's disease, NFTs = Neurofibrillary tangles and ACh = Acetylcholine. Created by BioRender.

1.2.4.1 Amyloid cascade hypothesis

APP is a type I single-pass transmembrane protein. It consists of three major parts. Extracellular ectodomain part binds metal and heparin. The middle part is the phospholipid membrane-permeating domain and the last part intracellular cytoplasmic tail (Polanco et al., 2018). It is found in many tissues and is concentrated in the synapses of neurons in the brain. It is essential for normal neuronal development and repair within the nervous system, where it is involved in cell growth, repair, and maintenance of synapses, which are essential for neuronal communication (Vassar et al., 1999). However, as Wilkins and Swerdlow have published in 2017, the function of APP is unclear (Wilkins and Swerdlow, 2017). In terms of the pathological amyloid cascade hypothesis of AD, the most important thing is to understand the amyloidogenic degradation process of APP, which results in the formation of unwanted extracellular plaques in the brain. From the point of view of pathological effects, the most significant factor is the disruption between the synthesis and clearance of Aβ (La Rosa et al., 2023).

However, in the context of AD, the APP becomes central to the pathogenesis of the disease and has a significant role. It undergoes many proteolytic degradation pathways by secretase enzymes. Conceptually, it has two major and significant cleavage processing pathways called amyloidogenic and non-amyloidogenic pathways. The process in which an unwanted and toxic fragment of

amyloid-beta is produced as a final product is called amyloidogenic, while the cleavage pathway that protects against production of Aβ is called non-amyloidogenic (Grimm et al., 2015).

Abnormal amyloidogenic processing of APP involving β-secretase and γ-secretase, leads to the production and accumulation of 4kDa Aβ peptides, including Aβ40 and the more toxic Aβ42. These peptides tend to aggregate and form extracellular senile and amyloid plaque deposits in the brain which is a hallmark of AD. The accumulation of Aβ plaques is thought to contribute to neurodegeneration and cognitive decline observed in the disease (Cras et al., 1991; Vassar et al., 1999). During the aggregation process of amyloid-beta, formed monomers bind to each other, forming soluble dimers and oligomer structures. These structures have a damaging effect on neurons and are toxic. For example, at synapses they inhibit reuptaking of excitatory glutamate thus leading to excessive neuronal activity. Ultimately, they bind to each other forming extracellular amyloid plaques in the brain leading to degeneration and death of nerve cells, shown precisely on figure 5. As a crucial neurotransmitter, the glutamate has an important role in memory. In pathology of AD, increased Aβ levels could affect amounts of glutamate in synapses and thus contribute to the progression of the disease (Kwon et al., 2020; Revett, et al., 2013).

During amyloidogenic cleavage pathway, APP is first cleaved by β-secretase enzyme (BACE1), which cleaves APP at the biologically longer and active NH2-terminal end of the Aβ peptide, releasing a soluble extracellular fragment (sAPPβ) and a shorter C-terminal fragment (CTF) on a cell membrane (Grimm et al., 2015).

β-CTF fragment, C99, is then cleaved by γ-secretase enzyme, resulting in the production of various lengths of amyloid-beta peptides, including Aβ40(1-40) and the more aggregation-prone Aβ42(1-42), along with the APP intracellular C-domain (AICD). (Grimm et al., 2015; Vassar et al., 1999).

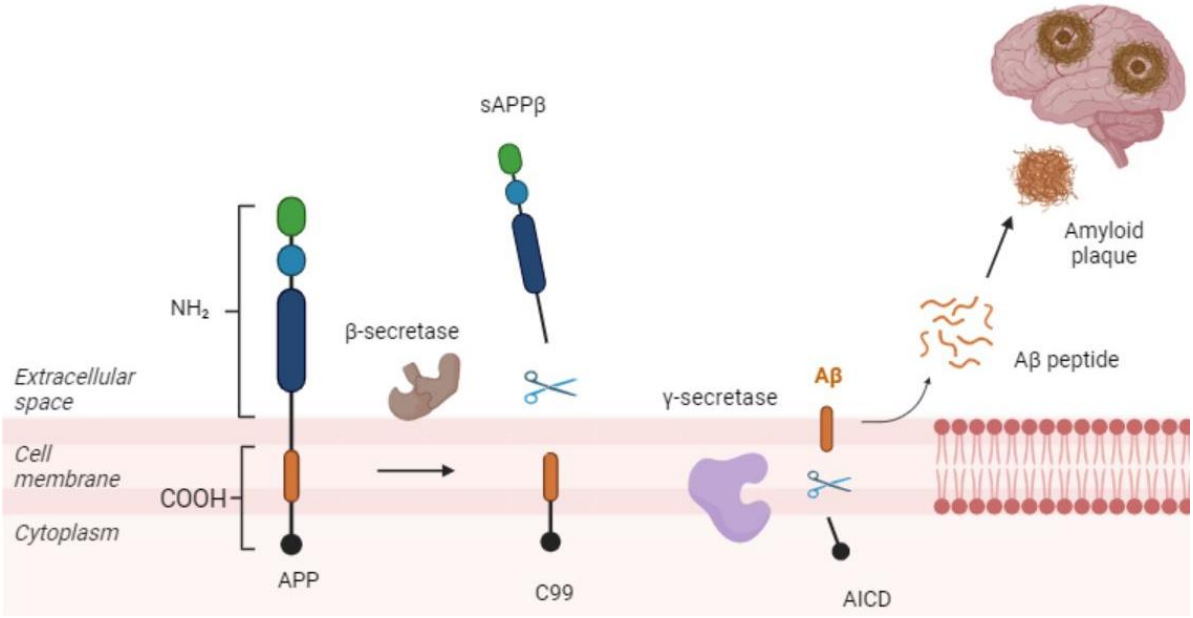

Figure 4. Amyloidogenic cleavage pathway. First β-secretase cleaves the APP at its extracellular and longer NH_2 terminal. This is followed by γ-secretase, which cleaves the central part of the remaining protein on the cell membrane leading to the release of Aβ peptides outside the cell. APP = amyloid precursor protein, sAPPβ = soluble extracellular fragment, Aβ = Amyloid-beta, C99 = COOH -terminal fragment of APP, and AICD = APP intracellular C-terminal fragment in cytoplasm. Created by BioRender.

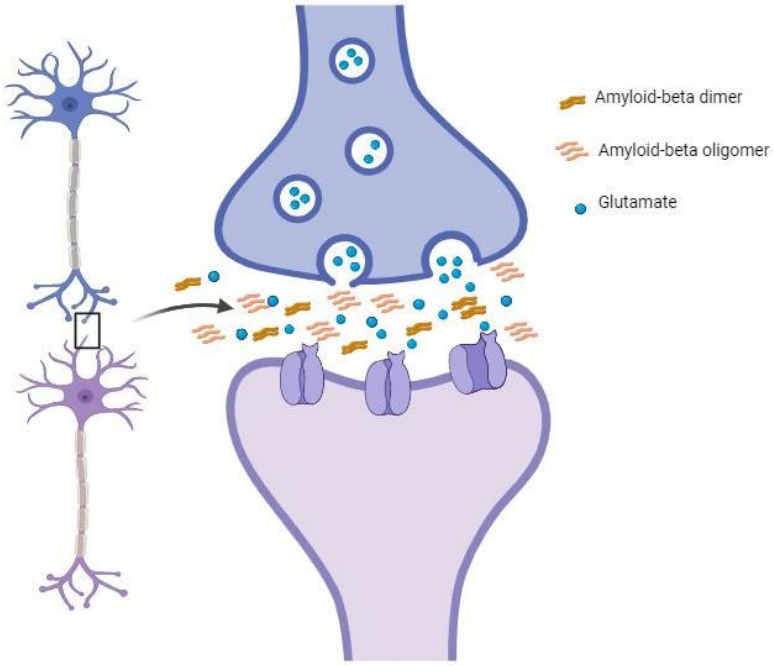

Figure 5. From amyloidogenic cleavage pathway formed Aβ monomers bind to each other forming amyloid-beta dimers and oligomers. At neuronal synapse, formed Aβ peptides inhibit and block retaking glutamate neurotransmitter causing thus disruptions in neuronal signaling. In a long-term excessive concentration, glutamate leads nerve cells into apoptosis and impaired brain function. Eventually causing damage and death of neurons in the brain. Created by BioRender.

The non-amyloidogenic pathway protects from formation of Aβ, as no toxic amyloid peptide is produced as that final product. In the APP cleavage pathway, α-secretase cleaves the APP protein

first at the site where Aβ is located, releasing the soluble extracellular sAPPα fragment and α-CTF, specifically C83. After this, the cleavage continues with γ-secretase, which cleaves the C83 fragment in the cell membrane, releasing the intracellular C-terminal fragment and the extracellular p3 peptide which is nonpathogenic as shown in Figure 6 (Vassar et al., 1999)

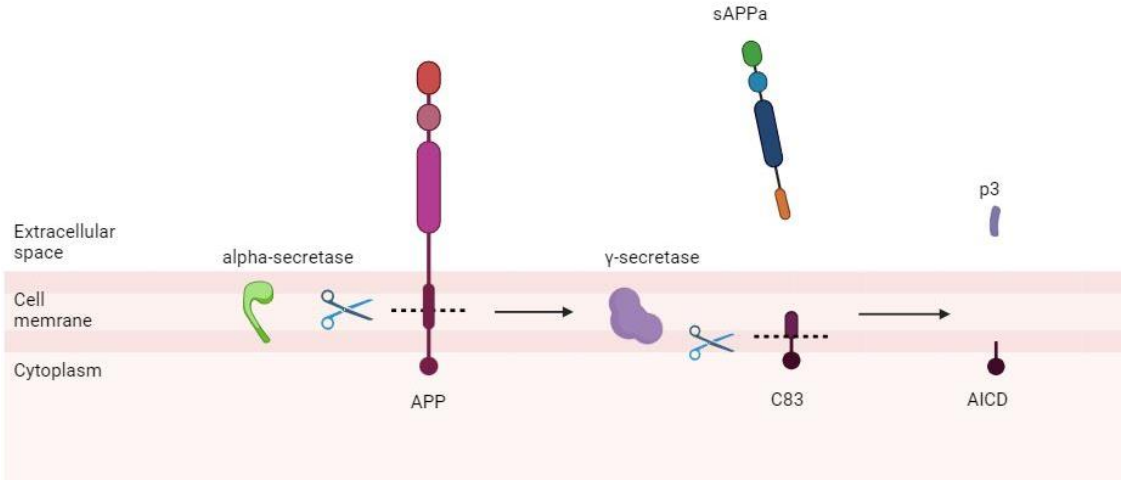

Figure 6. Non-amyloidogenic cleavage pathway. First α-secretase cleaves the APP at its intracellular site where the Aβ is located releasing sAPPα and C83. This is followed by γ-secretase which then cleaves C83 fragment releasing AICD and p3. APP = amyloid precursor protein, Aβ = Amyloid-beta, sAPPα = soluble extracellular fragment, C83 = Alpha-C-terminal domain, AICD = APP intracellular C-terminal fragment in cytoplasm and p3 = No toxic extracellular peptide. Created by BioRender.

1.2.4.2 Tau pathology

A microtubule-associated protein tau is a small intracellular 50 – 65 kDa protein that is related to the maintenance of neurons. It is mainly found in nerve cells, especially in the brain, where it regulates the stability and shape of microtubules, which are important structural elements of cells. Microtubules are essential for cell division, transport systems and signaling pathways (Delacourte and Defossez, 1986).

Under normal conditions the protein tau is protective in the brain, and it plays an important role in neurons stabilizing the microtubules and binding them together. Tau undergoes phosphorylation by protein kinases and dephosphorylation by protein phosphatases. The balance between these is important for the normal regulation of tau function and adaptation to the structural needs of the cell. In this way, it supports the functionality of the nerve cell in signaling and cell structure, allowing normal structural functions and transport of mitochondria and synaptic vesicles (Goedert, 1993).

Phosphorylation, where phosphate groups are added to tau by protein kinases, is important for intracellular transport. This allows tau to move within the cell and detach from the microtubule.

This in turn is followed by dephosphorylation, where the phosphate groups are removed from tau by protein phosphatases. Dephosphorylation guarantees the return of the tau to the microtubules, where its task is to maintain the microtubule framework. These are normal important intracellular regulatory mechanisms. However, in tauopathy, e.g., frontotemporal dementia and AD, tau is abnormally hyperphosphorylated and released from microtubules. The abnormal reaction balance between kinases and phosphatases eventually leads to the breakdown of microtubules and inhibition of cell flow promoting thus neurodegeneration (Soeda and Takashima, 2020).

At the cellular level, hyperphosphorylated tau starts to form paired helical filaments (PHFs), which in turn are components in the formation of toxic neurofibrillary tangles considered as a postmortem hallmark in the brain of AD patient. These NFTs, intracellular hallmark of the disease, are formed in the nerve cells where neurodegeneration occurs. They disrupt normal cell function and thus are associated with neuronal damage and death. Disease severity and dementia are correlated depending on the location and extent of these lesions in brains (Goedert, 1993; Kwon et al., 2020).

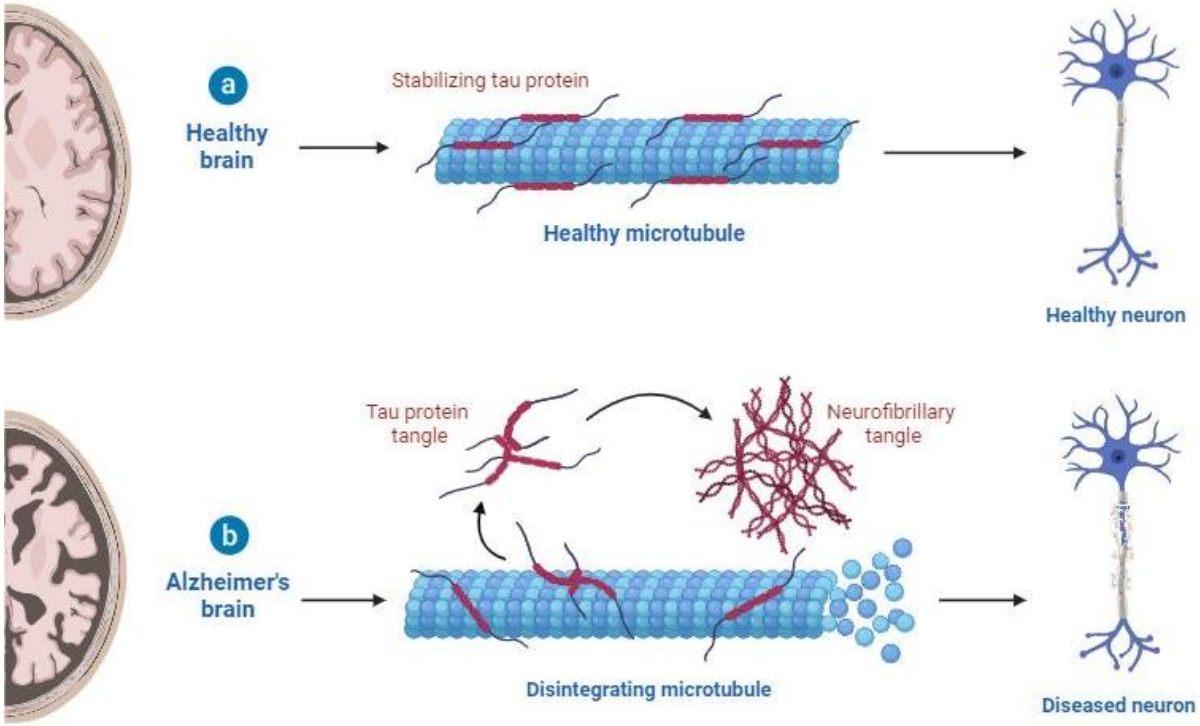

Figure 7. Tauopathy. The upper image a) shows a microtubule of a healthy neuron, where the tau protein is in normal state. The lower image b) shows the hyperphosphorylated tau protein, where its microtubule protective effect is disturbed. As seen in the figure, hyperphosphorylated tau detaches from the microtubules leading to disintegration of microtubules and blockage of cell communication. Tau proteins begin to adhere to each other forming oligomers, which in turn are toxic to many cellular organs. Hyperphosphorylated tau leads to the formation of aggregated tau protein tangles, which eventually stick together to form intracellular NFTs and thus cause degeneration of the nerve cell. NFTs = Neurofibrillary tangles. Created by BioRender.

Tau protein and its dysregulation are strongly related to the pathophysiology of AD and are central research targets in the effort to develop disease modifying treatments for this neurodegenerative disease. In the brain, protein phosphatase 2A (PP2A) is the most important phosphatase in the cell, which participates in the dephosphorylation of the tau protein. Decreased levels of PP2A lead to abnormal phosphorylation (Goedert et al., 1992).

Thus, the experimental part of this study is emphasized on PP2A and its activation by using midline 1 (MID1) -inhibitors. The importance of phosphatase is examined in more detail in the chapter 1.4 Function of protein phosphatase 2A.

1.2.4.3 Inflammatory pathology

Prolonged and chronic inflammation is harmful to the brain. This is known to release harmful cytotoxins that are detrimental to brain function, promoting neuroinflammation. Here, the microglial cell, an immune cell, plays an important role in the innate immune defense of the CNS. Under normal conditions it has anti-inflammatory and neuroprotective properties. It cleans the brain by phagocyting waste products and protects the brain from harm caused by tissue damage (Heneka et al., 2015; Ozben and Ozben, 2019). According to recent findings, inflammation of the nervous system is a key part of the pathologic mechanism of AD. Microglial cells, which function in the innate immune system, appear to have a significant role in self-sustaining and prolonged neuroinflammation leading to degeneration and dysfunction of neurons eventually leading them death (Xu et al., 2016).

Changes in the environment of microglial cells, such as misfolded proteins, inflammation, cytotoxic factors, and protein aggregates affect their function and activity. Upon encountering a change, an activated microglial cell neutralizes the changes and return to a protective quiescent state. However, constantly repeated stimuli such as the occurrence of Aβ aggregation in AD increases the amounts of both astrocytes and microglia and impair thus protective property of microglia. Thus, the microglial cells remain in a state of activity that increases inflammation constantly, leading to the activation of the immune response and thus the release of inflammatory mediators. (Khoury et al., 1996; Solito and Sastre, 2012). Activated microglia cause a local inflammatory reaction, releasing cytokines (e.g., IL-1 and TNFα), complement proteins and reactive oxygen species (ROS) (Chen and Zhong, 2014). In AD, microglial cells destroy synapses through complement-mediated phagocytosis. With their C3 receptors, they attach to the C1q and C3b complement factors near the amyloid plaques. They also increase disease pathology by affecting insulin receptor substrate 1

(IRS-1). The inhibition of insulin signaling in the cell can lead to neurodegeneration due to tau degradation and Aβ aggregation (Breijyeh and Karaman, 2020; Hettich et al., 2014).

Through this mechanism microglia increase axonal damage in nerve fibers and thus contribute to neurodegeneration and disease progression (Goldgaber et al., 1989). Among the cytokines, IL-1 is known to increase, for example, the expression of the APP gene (Buxbaum et al., 1992). The latest research results similarly suggest that Aβ protein has indirect effects on neurons. It compromises their function by triggering a microglial reaction to the CD40 ligand, which in turn leads to the production of harmful and toxic molecules to the neurons (Tan et al 1999). In drug development, efforts have been made to curb the inflammatory state and increase this equilibrium state of microglial cells by acting via chemokine (C-X-C-motif) ligand 1, but without results (Bivona et al., 2023).

1.2.4.4 Oxidative stress pathology

Oxidative stress is one of the many pathological mechanisms of AD. In AD, it is related to a cellular imbalance of free radicals and antioxidants. The most important factors are ROS, which damage neurons in the brain. Malfunctions in the DNA of the cells or proteins lead to the death of the cells and thus to the progression of the disease. Free radicals are formed, for example, because of abnormal mitochondrial function, which in turn would contribute to Aβ production and thus increase the formation of toxic amyloid plaque (Chen and Zhong, 2014).

Oxidative stress is known to negatively affect accumulation of Aβ and tau hyperphosphorylation thus promoting neuronal and synaptic loss (Chen and Zhong, 2014). According to the latest studies, it is known that metal ions also influence Aβ aggregation. For example, zinc increases the secretases that cleave APP and thus promotes the production of Aβ (Liu et al., 2019). With age, the concentrations of copper (Cu^{2+}) and iron (Fe^{3+}) increase. The abnormal interaction of these metal ions and zinc (Zn^{2+}) with amyloid-beta contribute to the hyper metalation of the peptide. Aβ begins to produce hydrogen peroxide (H_2O_2) from oxygen (O_2) or biological substrates such as cholesterol. This leads to Aβ neuro toxicity as well as auto-oxidation (Bush, 2003; Opazo et al., 2002). The meaning and effect of the aluminum metal is disputed. According to the (Crapper et al., 1973), aluminum contributes to the neurofibrillary deterioration of the nerve cell. Instead, antioxidants are known to have a protective effect against cell oxidation. Numerous antioxidants such as vitamin E and ascorbate protect against these effects and prevent the accumulation of peroxides (Behl et al., 1994; Terry and Katzman, 1983).

1.2.4.5 Mitochondrial pathology

Aβ toxicity also influences mitochondrial pathology causing mitochondrial impairment and dysfunction such as oxidative phosphorylation and mitophagy that take part in autophagy. In addition, mitochondrial bioenergy and dynamics are also susceptible to disruption (Polanco et al., 2018).

Somatic mitochondrial DNA mutation affects the mitochondrial function by downregulating its electron transport chain (ETC). This leads to increased pathological mechanisms that are e.g., increased levels of ROS from mitochondria and its decreased oxidative phosphorylation. Together, all of these cause the function of synapses to deteriorate and lead to apoptosis, followed by neurodegeneration (Swerdlow and Khan, 2004).

1.2.4.6 Vascular and cellular damage pathology

Vascular and cellular damage increase the risk of developing AD. Risk factors include cardiovascular diseases, high blood pressure, obesity, high cholesterol, diabetes, and head injuries. CVDs weaken the oxygenation of the blood circulation, cause emboli and affect the blood vessels. These factors contribute to a weakened cerebral blood flow, increased deterioration of cognitive functions, and consequently affect nerve damage (Breijyeh and Karaman, 2020).

Obesity and a high body mass index (BMI) instead contribute to an increased risk of developing e.g., diabetes via insulin resistance. This leads to hyperinsulinemia and eventually chronic hyperglycemia. Obesity also causes an inflammatory state, where secreted proinflammatory cytokines promote insulin resistance and thus further hyperglycemia. Changes in insulin effects may influence the development of AD by increasing Aβ accumulation and decreasing tau degradation (Breijyeh and Karaman, 2020).

Thus, diabetes increases the risk of AD, which is probably due to a disturbance in insulin signaling (Hettich et al., 2014). See section 1.2.4.3 for more details. Long-lasting high blood sugar predisposes to other pathological effects of the AD disease, such as mitochondrial dysfunction (Breijyeh and Karaman, 2020).

1.3 Current drug therapy in Alzheimer's disease

AD is the most common reason for dementia and there is no effective treatment for this progressively destructive and severe symptom causing disease (Forlenza et al., 2010). Current drug

therapies combined with possible rehabilitation mainly alleviates the symptoms but do not improve the progressing memory impairment in AD. Instead, existing treatments relieve pain, alleviate, and improve cognitive symptoms, and increase mobility (Reith, 2018). Current drug treatments are unable to slow the progress of the disease and nerve cell destruction. Since the lack of effective treatment options, the need for disease-modifying drug therapies (DMTs) to slow the progression of NDs and delay their clinical course is huge (Passeri et al., 2022).

1.3.1 Acetylcholinesterase inhibitors

In AD, the levels of ACh and activity of choline acetyltransferase that synthesizes it are impaired. Destruction of ACh in neurons leads to attention and memory disorders, and it impairs cognitive functions and decision-making. By preventing the breakdown of ACh with acetylcholinesterase inhibitors, the cognitive disturbances that cause these symptoms can be eliminated (Briggs et al., 2016).

Currently, donepezil, galantamine and rivastigmine are used as an anticholinesterase inhibitor in the symptomatic treatment for mild and moderate AD. The aim of symptomatic treatment is to raise ACh levels and thus improve cognitive function and behavioral symptoms (Briggs et al., 2016).

Unfortunately, drug treatments only relieve cognitive symptoms and behavioral disorders, but do not prevent amyloid accumulation in the brain and thus inhibit the disease progressing. Aβ damages the neural pathway that uses ACh as a mediator.

1.3.2 N-methyl-D-aspartate antagonists

Excessive overstimulation of the neuronal N-methyl-D-aspartate (NMDA) glutamate receptor or excess exposure to the glutamate neurotransmitter leads to excitotoxic neuronal death. In this reaction, there is an excessive influx of calcium (Ca^{2+}) through the ion channel related to NMDA receptor. Increased levels of excitatory glutamate can cause neuronal toxicity and dysfunction, which in the course of time lead to cell death, inhibiting the receptor binding of glutamate and hence decreasing its elevated levels are a key position in drug treatment. Cell-damaging enzyme processes with free radicals have a destructive effect on cells and contribute to their death. This is caused by overstimulation of the NMDA-receptor (Lipton, 2005).

Another drug used to treat AD is the NMDA antagonist, memantine, which is a non-competitive blocker of the glutamate NMDA receptor (Briggs et al., 2016). As the many studies have shown, the

memantine can be combined with anticholinesterases, resulting in better clinical efficacy than anticholinesterases alone (Farrimond et al., 2012).

1.4 Function of protein phosphatase 2A

Protein phosphatase 2A belongs to the serine/threonine family, which has a total of four different types PP1, PP2A, PP2B, and PP2C. Of these, PP2A has the highest activity and is one of the most important phosphatases in the cell. It is a significant regulator in cellular signal transduction. It dephosphorylates proteins and other kinases in the cells and seems to have a major role in tau phosphatase stabilization especially in the brain. The PP2A seeks to stabilize tau return to microtubules via dephosphorylation. A reduced activity of the phosphatase PP2A enzyme has been observed in AD. This contributes to the hyperphosphorylation of both APP and tau, thus contributing to the overproduction of toxic effects such as Aβ and the formation of NFTs (Baskaran and Velmurugan, 2018; Gong et al., 1994; Wei et al., 2020).

The regulation of PP2A activity is complex and affects many biological processes. It has many functions in the cell (e.g., proliferation, division, apoptosis, metabolism, and regulation of protein activity). Its reduced levels also affect tau dephosphorylation and increase the phosphorylation of APP at its Thr668 site, ultimately leading to the formation of both hyperphosphorylated tau and Aβ. Altered PP2A activity is associated with various diseases. Its decreased activity has been studied in many cancers (e.g., prostate, thyroid, and breast), where its expression is generally reduced. Increasing its activity has been found to slow the growth of tumor cells. Understanding the function of PP2A is therefore important because dysregulation of its function leads to various intracellular disorders and diseases (Orlandella et al., 2019; Wei et al., 2020).

The active heterotrimeric PP2A holoenzyme complex consists of three subunits that determine its specificity and function (Figure 8). The trimeric form of PP2A consists of a 65 kDa structural A-subunit, a regulatory B-subunit, and a 36 kDa catalytically active C-subunit. It has also a dimeric form that consist of A and C subunits, but that core enzyme is not as effective than its trimeric holoenzyme form, where the B subunit plays a key role at dephosphorylation of tau and defining the substrate affinity of the holoenzyme (Goedert, 1993; Xu et al., 2008).

The A subunit determines the intracellular location of PP2A and acts as a structural scaffold. It also contributes to the integration of other subunits. Instead, more than 15 different types of B subunits are known. They determine which target proteins PP2A attaches to. In addition to substrate specificity, they regulate the phosphate activity of PP2A, forming different trimeric combinations

with other subunits, and its temporal function. From the point of view of tau, the B55 family of subunit B is the most significant. The C subunit is responsible for enzyme activity. It performs the removal of phosphates from phosphorylated proteins. It has an amino acid tail where Tyr307 phosphorylation is inactivating and Leu309 methylation is activating (Goedert, 1993; Kaur et al., 2016; Longin et al., 2007).

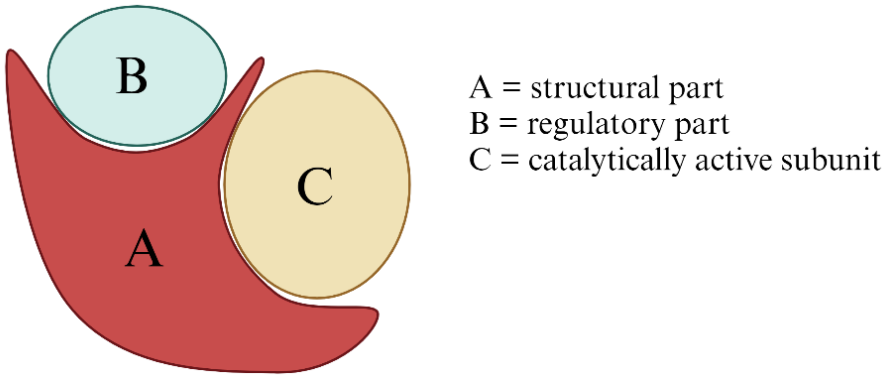

Figure 8. Trimeric structure of protein phosphatase 2A. Created by BioRender.

The A and C subunits have both two isoforms, α and β. Among the subunits, B is known to interact extensively with the α isoform of the A subunit but does not interact as much with the C subunit (Wei et al., 2020; Xu et al., 2008). However, the methylation and phosphorylation of C subunit play a significant role in which B-subunit of the PP2A complex associates with it. This interaction ultimately determines the function of the PP2A protein and its regulation. Interaction with post-translational modifications of the C-subunit and the B-subunit affects the functional specificity of PP2A and regulates its activity in various biological functions. This has been most studied in the B/55 subunit (Longin et al., 2007).

One of the regulators for PP2A is the α4 unit, which forms the MID1-α4-PP2A complex. MID1 regulates degradation of α4 and transports the B subunit to the proteasome for degradation. Correspondingly, MID1 inhibitors aim to prevent proteasomal degradation of the MID1-α4-PP2A complex on its α4 site, which would thus increase the amount and activity of the PP2A in the cells and increase cleavage of the α4 subunit (Du et al., 2013). The mechanism of action is shown more specifically in figure 9.

PP2A effect can be increased by drugs that activate it or inhibitors that would prevent its proteasomal degradation. In this study, small molecule MID1 inhibitors have been used to investigate the activity of PP2A. In the previous studies, (Kickstein et al., 2010; Schweiger et al., 2017) determinations have been made with non-specific MID1 inhibitors, such as metformin and

resveratrol, which have achieved good results against hyperphosphorylation of tau in animal cell models. More detail described in chapters 1.6.1.1 and 1.6.1.2.

1.5 The role of MID1 protein as a drug target

MID1 is a ubiquitin ligase protein, and it plays a significant role in cellular functions, particularly in microtubule regulation and PP2A modulation. Disruption of microtubule dynamics can impact cell division, migration, and intracellular transport. The binding of the MID1 protein, to microtubules, causes a ubiquitin-specific modification with the catalytic subunit of PP2A. This in turn leads to proteasomal degradation of PP2A. The process affects intracellular regulatory mechanisms such as phosphorylation and dephosphorylation, which play an important role in cell signaling and regulation (Schweiger et al., 2017).

As a drug target, MID1 has gathered attention due to its involvement in various cellular processes and its association with NDs. MID1 interacts with PP2A and by influencing PP2A function, MID1 becomes a potential target for therapeutic interventions aimed at restoring normal cellular processes. In the context of NDs, MID1 colocalizes with β-amyloid and tau in AD postmortem brain samples. This association is related to reduced PP2A activity, particularly linked to hyperphosphorylation of tau. Therefore, one promising target as a new PP2A activator, especially in NDs, is the MID1 protein (Schweiger et al., 2017; Sontag and Sontag, 2014). Targeting MID1 to modulate PP2A activity could influence tau phosphorylation and mitigate its pathological effects. Therefore, the purpose of this thesis work is to investigate how MID1 inhibitors affect the levels of PP2A, offering a potential avenue for disease modifying therapeutic development.

MID1 protein promotes the production of the mutant Huntington's(HTT) protein by binding to the S6K protein on the mutant HTT mRNA. At the same time, it inhibits the function of the PP2A protein and activates the mammalian target of rapamycin (mTOR) protein. Thus, drug therapy directed at the MID1 complex could inhibit the production of the pathogenic mutant HTT protein, which could be useful in the treatment of Huntington's disease (Krauss, 2016). Therefore, several mechanisms of toxicity in NDs, such as tau phosphorylation, APP cleavage, and mutant HTT protein synthesis in HD could thus be mediated through MID1 by using inhibitors (Du et al., 2013; Krauss, 2016).

Furthermore, MID1 plays crucial role in embryonic development implying that the appropriate level of the MID1 expression is significant for normal development of ventral midline structures. So, investigating MID1 as a drug target is relevant for developing therapies of genetic conditions such

as X-linked Opitz G/BBB syndrome that is related on mutations in the MID1 gene on the X chromosome. Mutations in the MID1 gene can affect the function of the MID1 protein and lead to dysregulation, especially for the MID1-α4-PP2Ac complex. This abnormal regulation promotes cell growth and development, manifesting as signs and symptoms of Opitz G/BBB developmental syndrome (Aranda-Orgillés et al., 2008; Baldini et al., 2020; Unterbruner et al., 2018).

MID1 has also been implicated in certain cancers, and its overexpression is observed in various malignancies. Targeting MID1 could be explored as a strategy to modulate cellular pathways involved in cancer progression. Namely, MID1 seems to mediate the ubiquitin-dependent degradation of α4, which affects the stability and activity level of α4. This process is a key factor when looking at the abnormal expression of α4 and its involvement in cancer processes (Du et al., 2013).

In conclusion, MID1 appears to be a multifunctional protein with essential roles in disease development, and potential therapeutic interventions, particularly in the context of NDs and cancer. MID1 has emerged as a potential drug target due to its involvement in various cellular processes. Its effect on degradation of PP2A is a significant factor. This affects the phosphorylation of tau, which regulation plays a central role in the treatment of AD. According to the hypothesis, disrupting the MID1-α4-PP2Ac complex with MID1 inhibitors is indeed a potential target for drug development.

The possibility of the MID1 as a drug target is increased also by previous studies (Krauss, 2016), where removing the MID1 gene from adult tissue of mice does not seem to have any vital adverse effects. It does not reduce the mouse's viability and would only cause mild motor learning problems. This suggests that MID1's functions extend beyond embryonic development and are relevant in the context of adult health and disease. Therefore, this would indicate that in a developed or aged body, MID1 modulation does not cause serious side effects, which would enable its use as a drug development target (Lancioni et al., 2010; Unterbruner et al., 2018).

1.6 MID1 inhibitors

The expression of MID1 in AD post mortem tissue is elevated, indicating that it may contribute negatively to decreased PP2A levels and elevated tau phosphorylation. The MID1 inhibitor could have an important role since it tends to increase the activity of PP2A protein phosphatase and thus increase its protective effect, allowing tau to return to the microtubules and preventing the formation of the combined PHFs formed from it (Baskaran and Velmurugan, 2018; Schweiger et al., 2017). However, no specific MID1 inhibitor have been published in Espacenet data bank.

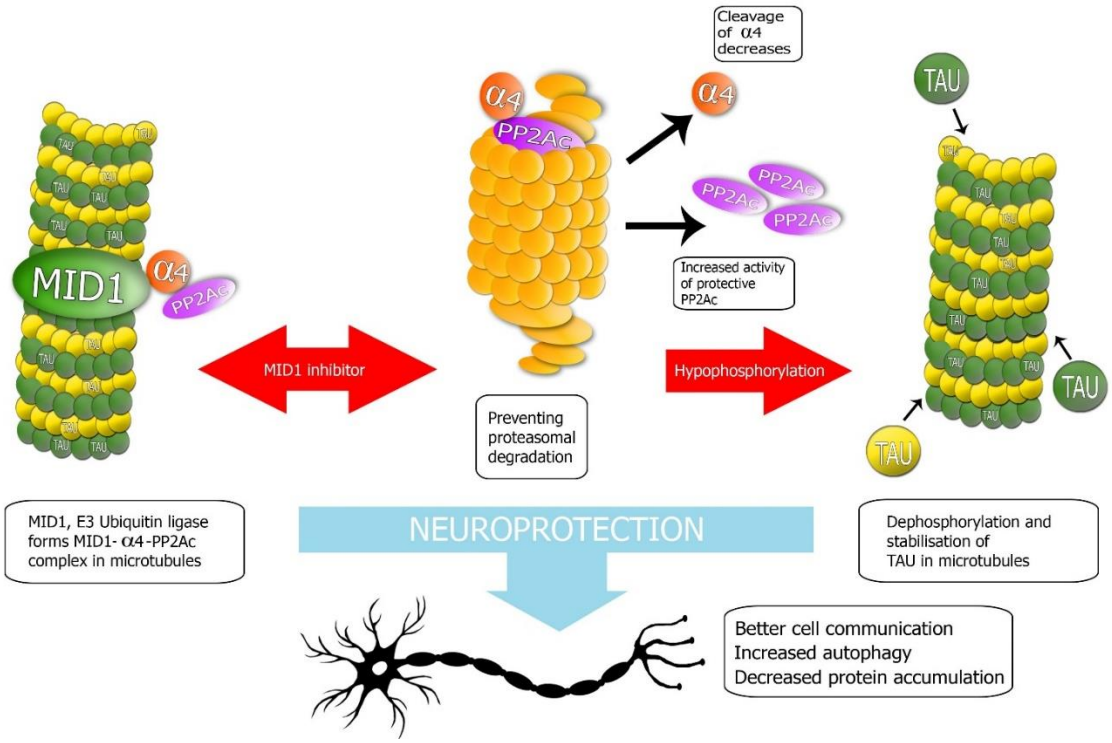

Figure 9. The mechanism of action, which describes the effect of MID1 inhibitors in preventing proteasomal degradation of the MID1-α4-PP2Ac complex. As a result of this, the protective effect of PP2Ac on microtubule-stabilizing tau proteins increases and the cleavage of the α4 subunit decreases.
As a result of dephosphorylation, the stabilization of tau in microtubules increases neuroprotection, where the communication between neurons improves, the accumulation of toxic proteins decreases and autophagy increases. Created by Nahawand Baski

1.6.1 Non-specific MID1 inhibitors

1.6.1.1 Metformin

Metformin is a drug commonly used to treat type 2 diabetes. It also has several potential effects in the body and has been of interest in studies of neurological diseases such as AD and PD. Studies suggest that metformin acts by increasing PP2A activity and thus has a decreasing effect on tau protein phosphorylation. This effect is mediated by metformin's ability to disrupt the binding of PP2Ac to the MID1-α4 complex. Disruption of the MID1-α4-PP2Ac complex with metformin reduces BACE1 expression via the mTOR kinase pathway, which reduces APP cleavage and β-amyloid formation (Hettich et al., 2014; Kickstein et al., 2010; Matthes et al., 2018).

In PD models, metformin reduces the amount of aggregation-prone Ser129 phosphorylated α-syn in cells and in the brain of mice, which is one of the most common findings in PD. This metformin-induced reduction is related to the activation of PP2A and inhibition of mTOR (Pérez-Revuelta et al., 2014).

In studies, metformin serves as a good positive control for MID1 inhibitors. It affects both mTOR signaling and α-syn dephosphorylation, which may be directly related to the interaction between MID1 protein and PP2A. This mTOR/PP2A signaling regulatory network gives metformin the potential to influence intracellular signaling mechanisms and protein regulation and may be a significant factor in the treatment of NDs (Hettich et al., 2014; Pérez-Revuelta et al., 2014).

1.6.1.2 Resveratrol

Resveratrol, a natural polyphenolic compound, has been studied in NDs, where it has been shown to have protective effects on neurogenesis. The beneficial effects of resveratrol may be in part manifested by its ability to increase the activity of PP2A. This effect is mediated by interfering the MID1-PP2A complex, which is ultimately followed by dephosphorylation of tau thereby preventing the formation of PHFs. The most significant effect here is the observed ability of resveratrol to reduce MID1 expression. Previous studies demonstrate resveratrol's ability to reduce AB toxicity and its effect on tau phosphorylation support also its importance in researches (Schweiger et al., 2017). In (Zhou et al., 2021), resveratrol has obtained good results, where it improves memory and learning in experiments with mice.

1.7 Future aspects of drug development field

Drug development for AD is a complex and challenging process due to the multifactorial mechanisms of the disease pathology and drug targets are not known precisely. These are also the biggest reasons for failures in the development of drug treatments in addition to lack of efficacy or safety issues. Various drug targets and compounds are explored to address the underlying causes and symptoms of AD, but more information is still needed on both the mechanisms of the disease and the mechanisms of action of the drugs (Athar et al., 2021; Cummings et al., 2019).

In the past, the research has mainly focused on Aβ as a drug target, but recently attention has shifted more to the tau targeting therapies, especially tau immunotherapies. These treatments are expected to achieve better efficacy in the treatment of cognitive symptoms compared to previous Aβ targeting treatments which have been ineffective (Congdon and Sigurdsson, 2018). In the future, medical treatment will move towards DMTs and multi-target therapy instead of monotherapy, aiming to affect multiple toxic mechanisms (Cummings et al., 2019; Menzies et al., 2017).
In Aβ-targeting drug development, the focus has been on influencing the formation of Aβ. Studies have included BACE1 inhibitors, gamma secretase inhibitors, and monoclonal antibodies such as aducanumab and lecanemab designed to reduce Aβ accumulation (Bazzari and Bazzari, 2022).

In tau targeting drug development, critical is to inhibit the formation of NFTs from abnormally hyperphosphorylated tau protein. There are several targets of action directed to tau, shown in the figure 7. Drugs aim to stabilize tau or inhibit its aggregation to prevent tau-related pathology (Bazzari and Bazzari, 2022).

As previously mentioned, many pathogenic mechanisms are known to contribute to the onset of the AD disease. The medical treatment of the future aims to investigate new drug targets and combination treatments that can treat several aspects of AD at the same time and that aim to change the disease instead of just alleviating the symptoms. Currently accepted drug treatments for AD are mentioned in chapter 1.3. The goal is to develop drugs that can effectively slow down the progression of AD, apply to several neurodegenerative diseases and bring significant clinical benefits. Continuous drug development work is central to advancing the understanding and treatment of AD (Athar et al., 2021).

1.8 MID1 inhibition could be one novel pathway for DMTs for AD

Although metformin and resveratrol have been shown to inhibit MID1 and good results have been obtained with these non-specific MID1 inhibitors, they are not suitable alone for the treatment of AD and PD due to their multiple targets and poor blood brain barrier permeability. Clinically, the effective concentration of resveratrol (10-100 μM) is difficult to achieve (Baur and Sinclair, 2006; Kickstein et al., 2010; Schweiger et al., 2017). Thus, a functional and developable MID1 inhibitor would be one that could allow the use of these PP2A activators at a lower and safer concentration, thus enhancing the synergistic effect (Pérez-Revuelta et al., 2014).

1.9 Purpose of the study

Molecular aberration-based theory is often associated with disruption of the intracellular ubiquitin-ligase proteasome system. This leads to changes in cellular backbone proteins, resulting in the accumulation of insoluble protein fragments or inclusion bodies in the cytoplasm or cell nucleus (Reith, 2018). In this master’s thesis the focus is on MID1-α4-PP2Ac protein complex system as PP2A activation is a tempting possibility for therapy, and MID1-α4 complex is responsible for its transport to proteasomes. The protein complex MID1-α4-PP2Ac is a potential target mechanism in drug development field, through which neuronal cell damage could be inhibited and thus the development of drug therapy to slow the course of the disease could be sought. By influencing on this protein complex mechanism with MID1 small molecule inhibitors, the purpose is to discover a therapeutic solution to the most common neurodegenerative diseases, such as Alzheimer's and

Parkinson's disease, and thus to influence and discover drug therapies that modify the course of these diseases.

Since, there is no effective treatment to retard neurodegenerative disorders the goal is to develop the medication to change the course of the disease. PP2A affects several mechanisms of toxicity, which is important in the treatment of neurodegenerative diseases, so the goal is to develop optimal MID1 inhibitors for further development as a novel PP2A activator.

This drug development research project is related to most prevalent and well-known ND. The focus will especially be on AD and its tau pathological mechanism since experimental work on this master's thesis focuses on PP2A and its activation aiming to influence this pathological disease mechanism.

The aim of this is to characterize MID1 protein as a drug target with some commercial small molecule inhibitors and study how the MID1 modification affects PP2A levels. In other words, the inhibitors aim to prevent proteasomal degradation of the MID1-α4-PP2Ac protein complex and to determine its effect on PP2A levels, as its increasing levels tend to stabilize tau microtubules. This protein-drug mechanism concentrates on the first phase of drug development in vitro level.

2 Results

2.1 MID1 validation with siRNA

MID1 expression was silenced by using MID1 small interfering RNA (siRNA) silencer (#AM51333, ThermoFisher Scientific) with increasing concentrations which were 12.5 µM, 25 µM and 50 µM. Stealth RNAi™ siRNA Negative Control (#12935300, ThermoFisher Scientific) was used in siRNA transfection as a negative control for comparing the results. Western blot and related preparations for it which included cell culturing, preparing well plates, siRNA transfection, cell lysis and determination of protein content, had to be repeated several times for MID1 protein validation and when testing commercial MolPort 4 and MolPort 6 small molecule MID1 inhibitor compounds. Despite all these attempts, experiments for the validation of MID1 protein as a drug target unfortunately failed.

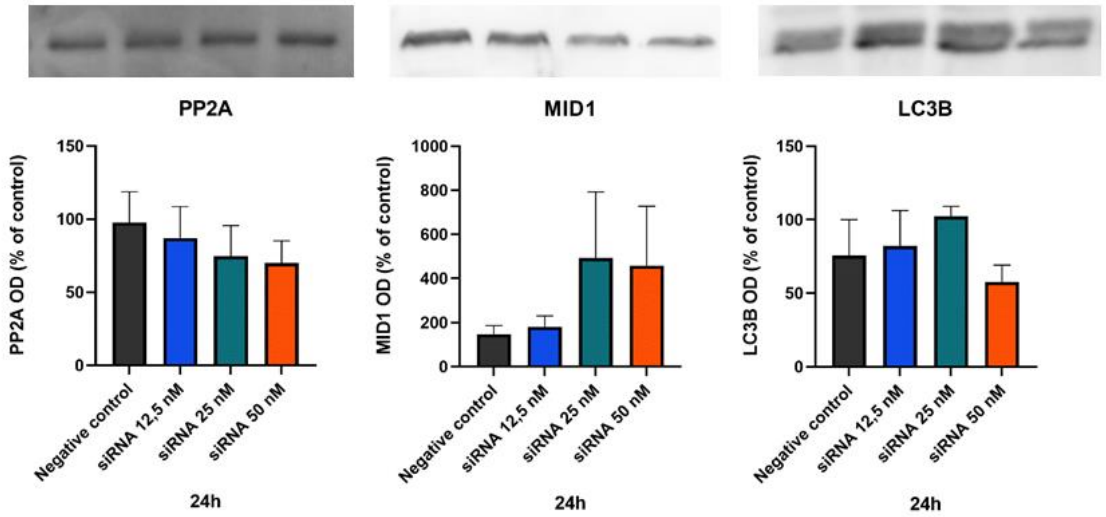

Figure 10. MID1 siRNA transfection results and silencing effect on MID1, PP2A and LC3B protein levels at 24h time point observation.

According to figure 10, it can be concluded that the silencing of the MID1 protein has not been successful. As the concentration increases, the protein concentration of MID1 protein increases in 24 h, as well. The amount of MID1 protein also does not decrease at the time point of 48 h which is observed from the figure 11 below. Huge dispersion could also be seen in the changes of PP2A. From the results, it can be concluded that the MID1 siRNA assay did not work and thus silencing the MID1 protein did not obtain the expected results. This means that the MID1 as a drug target protein, must be re-validated.

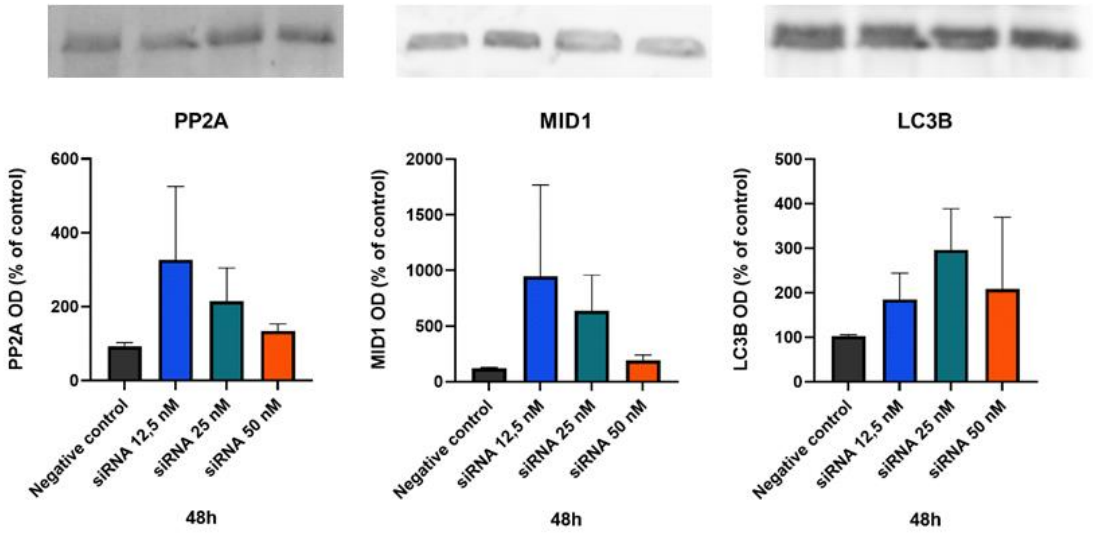

Figure 11. Levels of PP2A and MID1 proteins and LC3B control from Western blot based on MID1 siRNA transfection assay at 48h observation.

The levels of PP2A were decreased in both time observations. According to the One-way ANOVA results, the p value for PP2A at 24h was 0.7558 and at 48h observation p = 0.3672. Both p values are > than 0.05 which means the results are not significant.

2.2 Commercial MID1 inhibitors – effect on PP2A levels

These model MID1 inhibitor compounds MolPort4 and MolPort6 used in this study have been ordered from the MolPort Inc. (Latvia) and the compounds are based on modelling and the binding site of the MID1 protein.

2.2.1 MolPort 4

The effect of MID1-inhibitors on PP2A levels were tested in MID1 siRNA transfection in HEK-293 cells. MolPort 4 was tested at two concentrations which were 100 µM and 200 µM. The compound crystallized at both concentrations and proved to be toxic to the cells after both 24 h and 48 h incubation times as it can be seen in figures 12 and 13 below.

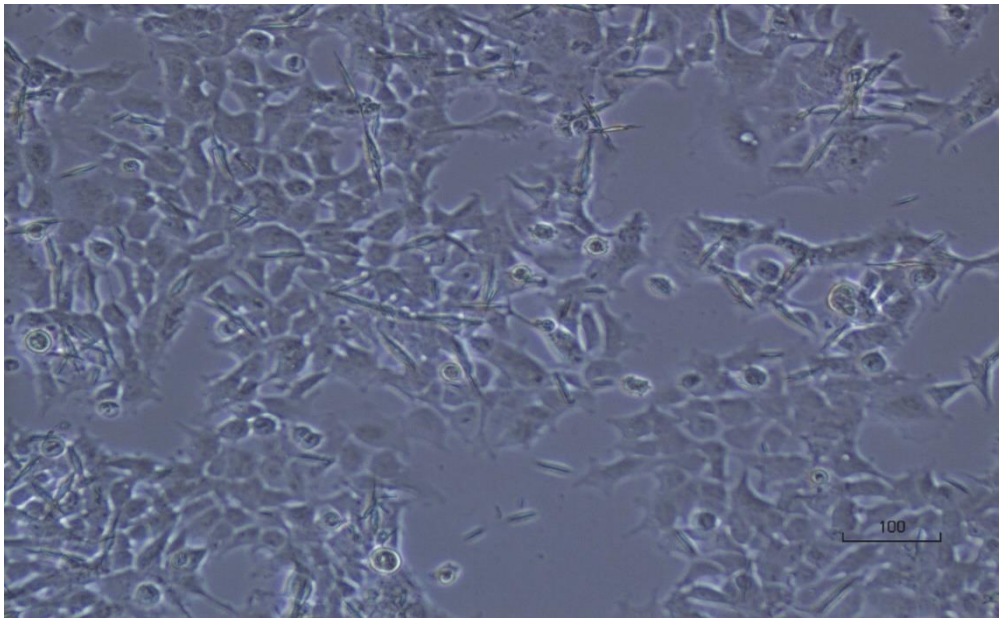

Figure 12. Crystallization of MolPort 4 with 100 μM in 24h observation for HEK-293 cell.

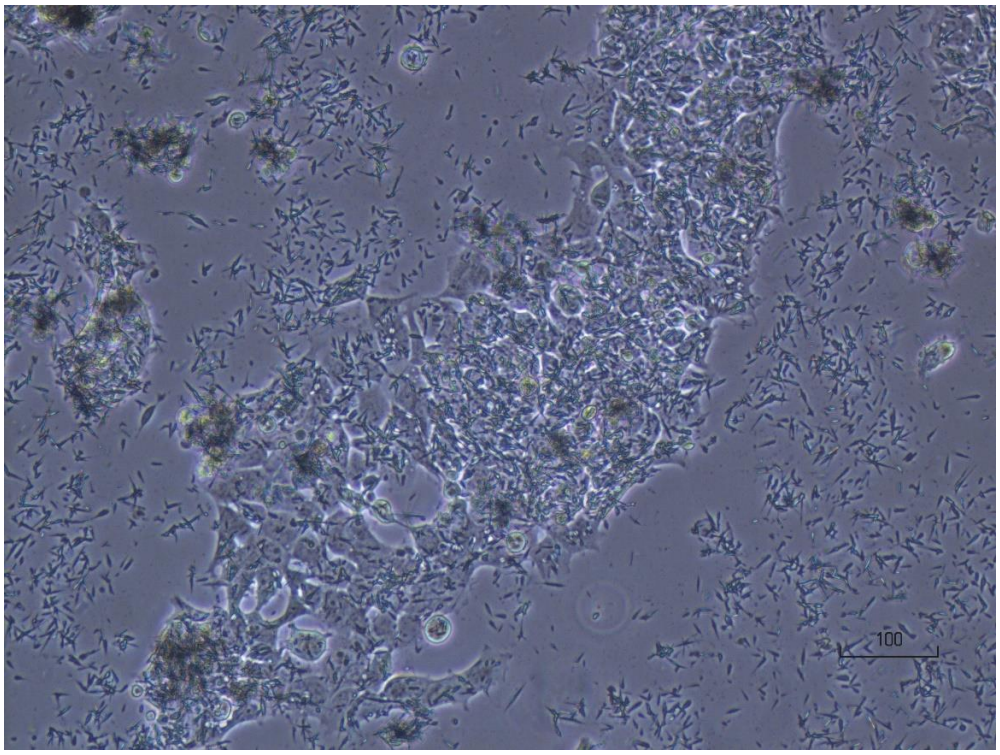

Figure 13. Crystallization of MolPort 4 with 200 μM in 48h observation for HEK-293 cell.

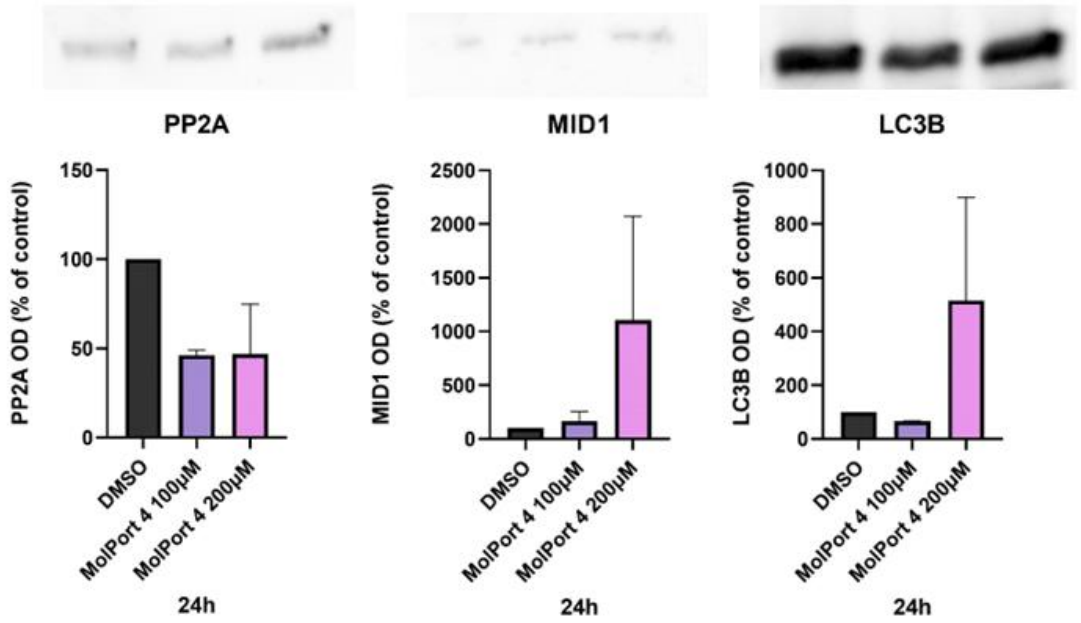

Figure 14. Levels of PP2A protein from Western blot based on MolPort 4 at 24h observation.

The results show that as the toxicity and incubation time increase, decreased levels of PP2A and LC3B protein can be observed. p(PP2A) = 0.4092; p(MID1) = 0.6274 and p(LC3B) = 0.5596. All the p values are not significant, $p > 0.05$. (Figure 14).

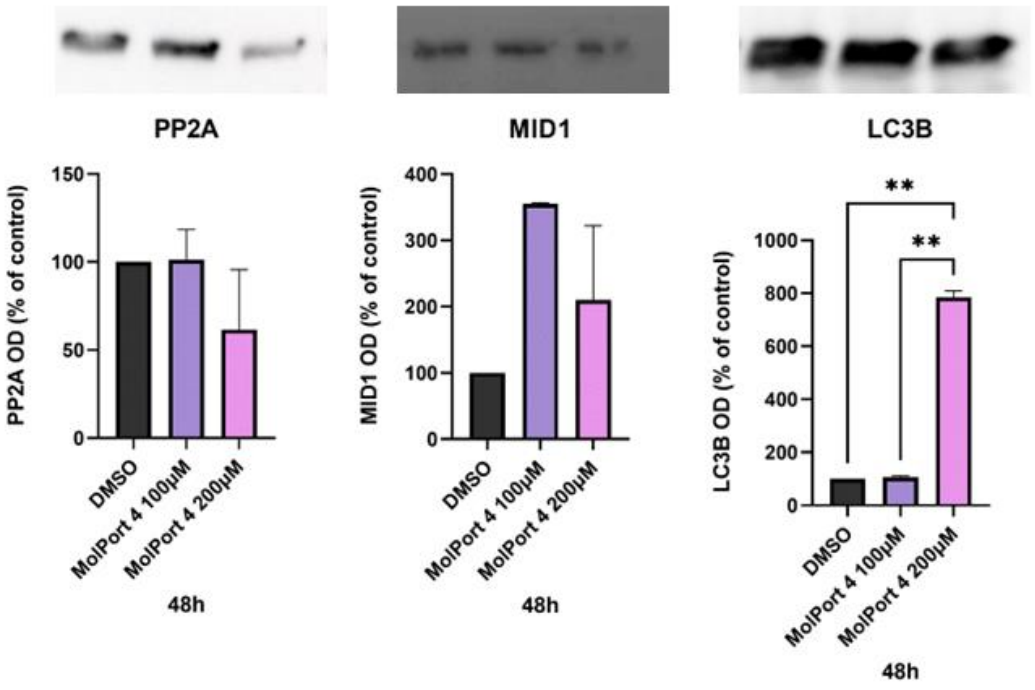

Figure 15. Levels of PP2A protein from Western blot based on MolPort 4 at 48h observation.

p(PP2A) = 0.6096; p(MID1) = 0.3441 and p(LC3B) = 0.0021. For LC3B which is autophagy marker the p value gave significance value $p < 0.05$. At a concentration of 100 µM, the LC3B value decreases, but at 200 µM it increases. Interpretation is made difficult by the toxicity of the compound. Shown in the figure 15.

2.2.2 MolPort 6

The levels of PP2A were seen increased when tested MolPort 6 compound. Increasing are observed with concentrations of 10 µM, 50 µM and 100 µM. Toxicity occurred at the highest concentration, 200 µM and thus a decrease in PP2A and MID1 proteins is seen. At other concentrations, an increasing dose-response is observed, as shown in the figure 16 below.

The levels of PP2A significantly increases $p = 0.0014$; ($p < 0.05$).

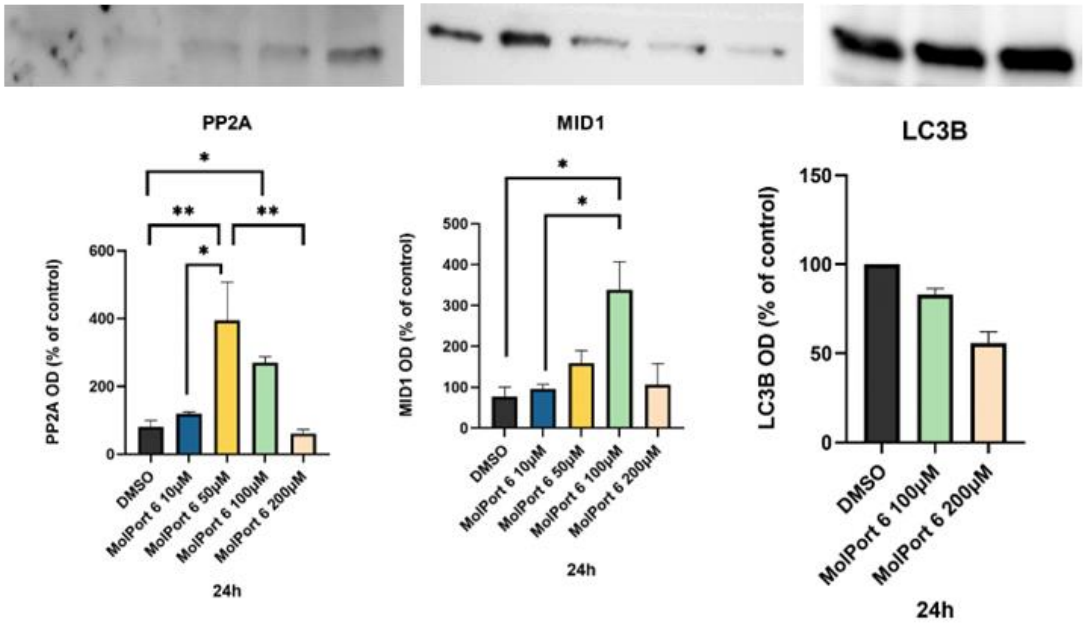

Figure 16. Levels of PP2A protein from Western blot based on MolPort 6 at 24h observation.

3 Discussion

In the study, we failed to perform MID1 validation with siRNA silencing. This can be explained by the lack of effect of siRNA transfection, which does not result in silencing MID1 expression. Moreover, the tested compounds had variable efficacy on MID1 or PP2A. It is likely that high concentration led to toxicity via crystallization, and this explains their inability to affect the MID1-a4-PP2A complex.

Although the results in siRNA transfection are not statistically significant in terms of MID1 effect and PP2A levels, they are indicative and the connection between them is noticeable with the MolPort 6 inhibitor compound, which appeared to be less toxic. According to what Baskaran and Velmurugan have published in 2018 PP2A promotes the protective effects of NDs, such as stabilizing tau and α-syn, as well as affecting autophagy, cell stress regulation and glial cell activation. Therefore, these results increase the potential for the MID1 protein to act as a drug target and the question of whether its silencing significantly increases these desired effects are mediated through the PP2A (Baskaran and Velmurugan, 2018).

Direct small molecule activators of PP2A like DBK-1160 are effective in AD's disease models (Wei et al., 2020), but sufficient brain penetration requires a high dose and their therapeutic index is narrow. This raises the question of whether MID1-mediated PP2Ac activation could be a more straightforward and safer target and a way to affect the activators that act directly to regulate the phosphatase and its complexes. Previous studies have also investigated the effects of the prolyl oligopeptidase (PREP) and its small molecule inhibitors in models of NDs. KYP-2047, a PREP inhibitor, has been found in studies to reduce the accumulation of tau protein and α-syn (Eteläinen et al., 2023; Savolainen et al., 2014). However, the efficacy of this KYP-2047 as a PP2A activator, which proved to be safe, was no longer sufficient in the PD's disease model. So, this tells us that the development of PP2A activators still has a long way to go before clinical applications. On the other hand, many PP2A activators act also indirectly, but they have multiple other targets or excessive toxicity in terms of NDs (Sangodkar et al., 2016), making them unsuitable treatment as a monotherapy. So, this entity also awakens the direction to move forward in the treatment of NDs towards multi-target thinking in field of drug development where several toxic mechanisms can thus be affected.

According to the results obtained from this study, the tested MolPort compounds 4 and 6 do not significantly affect PP2A levels, $p>0.05$. It can also be concluded from the results that they predict poor MID1 affinity and specificity. This means that the tested and proven to be toxic MolPort 4 and MolPort 6 inhibitors will need compound modification. On the other hand, for research purposes, new CNS-penetrating hit-inhibitor compounds produced by computer-aided drug design and synthetic chemistry will be needed, which will then be tested again in cell models of neurodegenerative diseases. As with drug development, the goal is to eventually find 5-10 suitable and effective lead-compounds that would be better suited for further trials.

Although this thesis did not achieve significant findings in the levels of PP2A, in summary, it can be concluded that MID1 as a new drug target for increasing the activity of PP2A with small molecule inhibitors offers a potential direction and sheds a light in drug development. Whereas direct and indirect PP2A activators have proven to be weak, MID1 would be suitable as a new research target for the development of treatments that affect the course of the disease. Due to its multifunctional property, MID1 would be suitable not only for ND diseases but also for other diseases mentioned earlier in the thesis, such as cancer.

4 Materials and methods

4.1 Cell culture

The HEK-293 cells used in cell culture, were fed with Dulbecco's Modified Eagle's growth medium (DME, DMEM; #D6429, Sigma® Life Science, 500 ml) to which 55 ml 10 % (v/v Fetal Bovine Serum, FBS pH 7.4, Gibco®; #2131514, 500ml) was added as a nutrient and an 5 ml 1 % (v/v Penicillin 10,000 Units/ml – Streptomycin 10,000 µg/mL, Gibco®; #2199829, 100 ml) antibiotic mixture solution for inhibiting microbial growth. Trypsin-EDTA (0.05 %, Gibco®; # 25300062,) was used in cell division to detach cells from the bottom of the culture medium. The cells were separated from the supernatant which consists of a little amount of trypsin-EDTA and medium, by centrifuging the cell suspension in the falcon for three minutes at 1600 rpm by using Sigma Centrifuge apparatus Rotor Nr. 11133, 5500/min. After separation, the supernatant was discarded, the cells suspended in a small amount of medium, and the number of live cells determined by making a total of 20 µl a 1:1 ratio mixture of cell suspension and Tryptan blue detection agent using Bio-Rad TC20™ Automated Cell Counter. The dye used in cell counting can be used to count the number of living and dead cells. Tryptan blue works in such a way that in living cells it is not able to penetrate the cell membrane, but in dead cells it is able to get inside the cell.

The 12-well plates were prepared based on cell counting. The amount of cell suspension to be pipetted was obtained by calculating the desired number of cells divided by the determined number of living cells (Table 2).

Table 2. The following cell values have been obtained for making well plates:

Live count (cells/ml)	Volume on 24h-well plate (100 000 cells)	Volume on 48h-well plate (80 000 cells)	Purpose
$3.42x10^6$	29.2 µl	23.4 µl	siRNA transfection
$8.40x10^6$	12.0 µl	10.0 µl	siRNA transfection
$3.88x10^6$	26.0 µl	21.0 µl	siRNA transfection
$5.68x10^6$	17.5 µl	14.0 µl	siRNA transfection
$9.07x10^6$	11.0 µl	9.0 µl	MolPort 4&6testing)

Live count (cells/ml)	Volume on 24h-well plate (200 000 cells)	Volume on 48h-well plate (160 000 cells)	
$4.30x10^6$	46.5 µl	37.5 µl	MolPort 4&6 testing
$3.73x10^6$	54.0 µl	-	MolPort 6 testing

4.2 Validation of MID1 as a drug target by using siRNA

For MID1 siRNA transfection, HEK-293 cells were seeded 1 x 10^5 and 0.8 x 10^5 in 12-well plate for each well with 1 ml Dulbecco's Modified Eagle's growth medium (DME, DMEM; #D6429, Sigma® Life Science, 500 ml) was added 5 ml 1% (v/v Penicillin 10,000 Units/ml – Streptomycin 10,000 µg/mL, Gibco; #2199829, 100 ml) solution and 55 ml 10% (v/v Fetal Bovine Serum, FBS heat inactivated, pH 7.4, Gibco; 10500064).

For 24h-well plate amount of cell was 100 000 and correspondingly for 48h-well plate 80 000 cells. The cells were incubated at 37 °C in 5.4 % CO_2 overnight. MID1 Silencer® Validated siRNA (ThermoFisher Scientific, Ambion; #AS02GCMT, 20 nmole) was used for silencing MID1 expression with increasing concentrations. 400 µl nuclease free water was added to 20 nmol siRNA and thus diluted 50 µM stock was prepared.

Next, RNA-lipid complexes and dilution series of siRNA were prepared and pooled together prior to addition to the 24h and 48h well plates. Lipofectamine® RNAiMAX, a transfection reagent (ThermoFisher Scientific; #2209739, 0.3ml) was diluted in Opti-MEM® Reduced Serum Medium (Gibco; #2241456, 500ml) accordance with 2 µl: 100 µl ratio. For two 12-well plate transfection, 26 µl lipofectamine RNAiMAX transfection reagent was diluted into 1300 µl of Opti-MEM medium.

MID1 siRNA dilute series was done from previously prepared 50 µM stock solution to using same Opti-MEM medium. The dilution series consisted of three different concentrations: 50 nM, 25 nM, and 12.5 nM with a ratio of 1:100 µl, and 0.5:100 µl and 0.25:100 µl. This means that, for 50 nM was taken 3 µl, for 25 nM 1.5 µl and for 12.5 nM 0.75 µl of MID1-siRNA 50 µM stock dilution which was diluted into same amount (300 µl) of Opti-MEM medium.

In addition, a negative control was prepared from Stealth™ RNAi Negative Control Duplex (#2160358, ThermoFisher Scientific) into the same Opti-MEM medium in a ratio of 1:100 µl, to validating and comparing the results. In this case, 3 µl negative control was diluted into 300 µl Opti-MEM medium. At the end, diluted lipofectamine RNAiMAX reagent was added to diluted MID1 siRNA in a ratio of 1:1, which meant amount of 300 µl to 300 µl.

After mixing the dilutions, 200 µl siRNA-lipofectamine complexes were added to each well, the plates were mixed gently and incubated for 10 minutes in room temperature before transferring them to a 37 °C incubator. Another of the well plates was incubated overnight for 24h and the other for 48h before cell lysis.

4.3 Testing novel MID1 inhibitors, MolPort 4 and MolPort 6 in HEK-293 cells

The synthesized MID1 inhibitor compounds, MolPort4 and MolPort6 were screened as activators of PP2A and observed how they affect the MID1-α4-PP2A complex using the same HEK-293 cells. The cells were seeded on two 12-well plates in 1 ml Dulbecco's Modified Eagle's growth medium (DME, DMEM; #D6429, Sigma® Life Science) with density of 2 x 10^5 cells per well for 24 h 12-well plate and 1.6 x 10^5 for 48 h 12-well plate. Both well plates were incubated at 37 ℃ in a 5.4 % CO_2 overnight.

When testing the MID1 inhibitors, dilutions of compounds were prepared from 100 mM stock in Sterile Dimethyl Sulfoxide (DMSO) diluting into Dulbecco's Modified Eagle's growth medium (DME, DMEM; #D6429, Sigma® Life Science). MolPort 4 was tested at concentrations of 100 µl and 200 µl. As the MolPort 4 was toxic to the cells, it was not further tested. Instead, MolPort 6 seemed more promising especially with 24 h timepoint and was tested at multiple concentrations which were 10 µl, 50 µl, 100 µl and 200 µl. DMSO served as a negative control as the compounds were prepared as 100 mM stock in DMSO. Well plates were incubated at 37 ℃ in a 54 % CO2 for 24 h and 48 h before cell lysis.

4.4 Cell lysis with RIPA buffer

To determine the proteins contained in HEK-293, cells were lysed with RIPA buffer and proteins were isolated from the cells. Modified RIPA buffer was prepared before cell lysis. The buffer was made of 50 mM Tris HCl 1.576 g ($C_4H_{11}NO_3$*HCl), 150 mM NaCl 1.75 g, sodium deoxycholate 0.5 g ($C_{24}H_{39}NaO_4$, D6750-10 g) and of 2 ml NP-40. The pH value was adjusted to 7.4 with NaOH. Halt™ 100 X protease and phosphatase inhibitor cocktails (#78440, ThermoFisher) was added to a proper amount of the RIPA solution for protecting proteins from degradation during cell processing.

For cell lysis, 1386 µl of RIPA buffer was added to the Eppendorf tube, to which 14 µl of protease and phosphatase inhibitor cocktails was added. The cells were washed once with 300 µl phosphate buffered saline (PBS pH 7.4 Gibco #2131514). After washing the cells, 100 µl of buffer-inhibitor mixture was added for 12-well plate. The cells were scratched and when pipetting the cell suspension to an Eppendorf tubes, they were broken three times up and down. The samples were centrifuged at 16,000 g in 4 ℃ and for 15 minutes using Eppendorf® Centrifuge 5424R or Hitachi himac CT 15RE centrifuge. Soluble fraction, the supernatant was collected carefully for protein assay, and the collected samples were transferred to the freezing. In short-term storage, the samples were frozen at -20 ℃ and in long-term storage they were transferred to -80 ℃.

4.5 Protein assay by BCA method

Commercial Pierce™ BCA Protein Assay Kit (#VI311369; ThermoFisher) was used for the determination of proteins before western blot. Standards were prepared from Bovine Serum Albumin (BSA) 2 mg/ml stock to RIPA buffer. The working reagent, BCA assay solution was prepared of Reagent A by mixing 50 parts of it with 1 part of Reagent B (reagents; A50 : B1). The sample dilutions were prepared by first pipetting 8 µl Milli Q water into 96-well plates and pipetting 2 µl of the collected supernatant from cell lysis.

Next, control BSA dilutions were pipetted 10 µl per each empty well and finally 200 µl of BCA assay solution is added to each well and the plate was incubated for 30 minutes at 37 °C. The well plate was allowed to cool for 3 minutes at room temperature, after which the absorbance was measured by Ensight™ Multimode Plate Reader by PerkinElmer using the BCA protein assay absorbance 562 nm protocol. The real protein concentration was counted based on the absorbance values.

4.6 Western blot

After MID1 silencing, the levels of total PP2Ac (phosphorylating subunit), methylated PP2Ac (active form) and phosphorylated PP2Ac (inactive) were studied by Western blot to assess the effect of MID1 on PP2A. Levels of B55 subunit of PP2A were also studied since B55 targets PP2A to tau, and autophagy markers LC3B and p62 were also studied by using Western blot.

Different solutions were used in western blot. Solutions were prepared by weighing dry substances to which MilliQ water is added. Before using or mixing the solutions, pH is adjusted to 7.4 by using HCl. Before using the solutions, they were diluted by taking 100 ml of each solution, which was dissolved in 900 ml of MilliQ water.

For running buffer, 1000 ml MilliQ water was added on 30 g Tris, 144 g glycine and 10 g Sodium Dodecyl Sulphate (SDS). Tris-buffered saline (TBS) which is based on phosphatase detection, was used as a blocking buffer and for the preservation of membranes that was prepared of 30 g Tris, 80 g NaCl, 2 g KCl and dissolved into 1000 ml MilliQ water. For washing the membranes and stopping chemiluminescence was used tris-tween buffered saline (TTBS) which was prepared by dissolving 100 ml of TBS in 900 ml of MilliQ water, to which is finally added 500 µl of Tween-20, also known as Polysorbate 20 (#SLBS7729, Sigma® Life Science). Transfer buffer, which was used for transferring the gel by using Trans-Blot SD, Semi-dry transfer cell (Bio-Rad), was made by

dissolving 100 ml of running buffer and 200 ml methanol in amount of 700 ml MilliQ water. The function of 5 % milk in TTBS was for blocking the non-specific epitopes and the milk was prepared by weighing 5 g of milk powder (#T145.2, Carl Roth) which was dissolved in 100 ml of TTBS. 4x Laemmli Sample buffer SDS (#1610747, Bio-Rad) was used to prepare the protein samples. The SDS which it contained, denatured the proteins, and gave them a negative charge. 4x Laemmli SDS was prepared by adding 100 µl of β-mercaptoethanol to 900 µl of 4x laemmli sample buffer.

When preparing the samples for western blot, amount of the needed samples as a µl were calculated for 20 µg or 30 µg of protein. MilliQ water was first pipetted into each Eppendorf, then a protein sample, and finally 4x laemmli SDS in a hood based on the calculated amounts. Prepared samples were boiled for 5 minutes at 96 ℃ degrees by Eppendorf® ThermoStat Plus. To obtain the total amount of samples, the Eppendorf were spun for 10 – 15 seconds with table spinner. SDS-PAGE technique was used and 30 – 50 µl of protein samples were loaded to commercial Mini-Protean TGX 12 % Stain-Free Gels (#4568044, Bio-Rad). Before assembling the gel cassettes into the Mini-Protean Tetra system, the gel tape was peeled off at the bottom of the cassette and the comb was removed. The prepared and diluted running buffer was added into the running module starting with the wells and inner chamber. The outside of the cassette is also rinsed, and the module is filled about halfway. The first well of the gel was pipetted with 5 µl Precision Plus Protein Kaleidoscope - marker (#1610375, Bio-Rad) or with PageRuler Plus Prestained Protein Ladder (#26619, ThermoFisher). After this preparation, the gels were run for 35 minutes at 200 volts (V). An electric current cause negatively charged proteins to run towards positive electrode. The protein components separate into fractions in the order of their molecular weight, with the heavier molecules remaining at the top of the gel and the smaller molecules run correspondingly faster to the below of the gel.

When using a nitrocellulose membrane, it was moistened with transfer buffer before transferring the gels with a Trans-Blot SD, Semi-dry Transfer Cell device (Bio-Rad). However, this device did not produce successful western blots results despite attempts and thus for the most part and because of the success, a commercial Midi Format, 0.2 µm nitrocellulose trans-blot transfer pack was used (#1704159, Bio-Rad) and the gels were transferred by Trans-Blot® Turbo™ Transfer System (Bio-Rad) for 7 minutes with settings 2.5 ampere (A) and 19 V.

Before adding the primary antibody, membranes were incubated for an hour on the plate shaker at room temperature in 5 % milk in TTBS, that was added 5 ml per membrane. The function of the milk was to prevent binding of non-specific epitopes into membrane. The purpose was to facilitate

the detection of the target protein, as the binding of the non-specific antibody to the membrane complicates the determination.

Because the method is based on SDS-PAGE, the membranes were cut at 50 kDa, where primary antibody MID1 at ratio of 1:1000 or 1:500 was placed on the top of the membrane and PP2Ac primary antibody at ratio of 1:1000, respectively was put on the bottom of the membrane. Only then the primary antibodies were added according to the table 3 below.

Table 3. Antibodies used in detail

Antibody	Species	Manufacturer	Product # Lot	Dilution	Additional information
PRIMARY					MW (kDa)
MID1	rabbit	ThermoFisher Scientific	PA5-38524 #VI3078981	1:1000 / 1:500	75
PP2Ac	rabbit	Cell Signaling	2259S #(52F8)	1:1000	36, 38
β-actin	mouse	ThermoFisher Scientific	MA5-15739 BA3R	1:2000	~ 42
LC3B	rabbit	Sigma-Aldrich	L7543 #049M4783V	1:1000	16–18
SECONDARY					
Goat Anti-Rabbit IgG, (H+L)	rabbit	ThermoFisher Scientific	31460 #VC297287	1:2000	Host/isotype Goat/IgG Concentration: 0.8 mg/ml
Goat Anti-Mouse IgG, (H+L)	mouse	ThermoFisher Scientific	31430 #UL295755	1:2000	Host/isotype Goat/IgG Concentration: 0.8 mg/ml

The primary antibody was added according to the dilution ratio shown in Table 3 and the membranes were incubated at + 4°C on the plate shaker overnight. The next day, primary antibodies were collected, and the membranes were first rinsed twice with TTBS and then washed three times for 5 minutes with TTBS at plate shaker for removing the unbound and non-specifically bounded antibodies. In order, to preserve the collected primary antibody for longer and, if necessary, to reuse, sodium azide (NaN3) was added as a preservative in a ratio of 1:500.

The membranes were then treated with a secondary antibody. The secondary antibody Goat Anti-Rabbit Horseradish peroxidase (HRP) (#31460, ThermoFisher Scientific) was used for all primary

antibodies except for β-actin to which respectively was used Goat Anti-Mouse HRP (#31430, ThermoFisher Scientific). Both secondary antibodies were made into 5 % milk in TTBS in a ratio of 1:2000 and added to 5 ml per membrane. Thus, 2.5 µl of secondary antibody is added to 5 ml of 5 % milk in TTBS. After the addition of the secondary antibody, the membranes were incubated for two hours in room temperature at plate shaker. The membranes were washed again before chemiluminescence imaging. They were rinsed first twice with TTBS and then washed twice for five minutes first with TTBS and then with TBS.

Beta actin was used as a control to determine the amount of protein. It was prepared in the same manner as mentioned above. β-actin was prepared in a 1:2000 dilution ratio to 5 % milk in TTBS and the membranes were incubated at + 4 ℃ on the plate shaker overnight. On the next day, β-actin was collected, and membranes were rinsed and washed with TTBS before adding secondary antibody. For β-actin, the secondary was made of Goat anti mouse HRP (#31430, ThermoFisher Scientific) that was prepared in a 1:2000 dilution ratio to 5 % milk in TTBS. Then, the membrane was incubated for 2 hours at plate shaker in room temperature. Membranes were rinsed and washed in the same manner before chemiluminescence imaging.

Also, an autophagosome protein marker, LC3B was done like β-actin, but it was prepared in a 1:1000 dilution ratio to 5 % milk in TTBS and the used secondary antibody for LC3B was Goat anti rabbit HRP (#31460, ThermoFisher Scientific).

All the proteins, MID1, PP2A, β-actin and LC3B were determined from the membrane with SuperSignal West Pico PLUS Chemiluminescent Substrate Kit (#34580, ThermoFisher Scientific) or with SuperSignal West Femto Maximum Sensitivity Substrate Kit (#34095, ThermoFisher Scientific) detection reagents. The membranes were imaged by Sapphire Biomolecular Imager (Azure biosystems). After Western Blot treatment, all membranes were dried so that they can be reused later if necessary.

4.7 Statistical analysis

Optical densities of proteins were determined from Western blot membrane images which were processed by ImageJ (version 1.53e) software. The images were typed for 8-bit and the image was transformed horizontally if needed. Thereafter, each band was delimited and bounded individually. As many lines were selected and marked as there were bands, and they were plotted as a histogram. The area of each histogram was outlined and closed, and the areas were calculated by using wand tracing tool. The values obtained were normalized and calculated against β-actin on Excel sheet. In

Western blot Excel analysis, the individual value of each protein was calculated separately by dividing the value by the corresponding value of beta-actin. For comparison, each protein value was also calculated as 100 %. When a large variation appeared in protein amounts, the average and standard deviation (SD) were calculated from the surveyed values. The SD was multiplied by two and it was subtracted or increased from the average. The value obtained is compared with the determined protein values, from which, if necessary, individual values are removed to facilitate degradation.

The final western blot values for each protein at the same time point were summarized in the GraphPad Prism for statistical analyses (GraphPad software, version 9.1.0). For column bar graphical statistical analysis was chosen Mean with SEM plot which reduces deviations. The columns presented (Mean ± SEM). Where on each definition the averages for negative controls were chosen and calculated as 100 percent, and so the variability between columns were reduced. At statistical analysis for comparing differences and variations between groups was used One-way ANOVA since there were analyzed 3 – 5 values. Tukey's multiple comparisons test was followed by the One-way ANOVA if the P value gave significant values ($p < 0.05$).

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6 Abbreviations list

Aβ	Amyloid beta
ACh	Acetylcholine
AD	Alzheimer’s disease
AICD	APP intracellular C-domain
ApoE4	Apolipoprotein E 4 allele
APP	Amyloid precursor protein
α-syn	Alpha-synuclein
BACE1	Beta secretase enzyme
CNS	Central nervous system
CTF	C-terminal fragment
DMSO	Dimethyl Sulfoxide
DMTs	Disease-modifying therapies or treatments
HD	Huntington's disease
HRP	Horseradish peroxidase
mTor	Mammalian target of rapamycin
NFTs	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
PD	Parkinson’s disease
PP2A	Protein phosphatase 2A
PSEN1	Presenilin 1
PSEN2	Presenilin 2
ROS	Reactive oxygen species

sAPPα	Soluble alpha secreted APP fragment
sAPPβ	Soluble beta secreted APP fragment
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
TBS	Tris buffered saline
TTBS	Tris-tween buffered saline

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