The Role of Alpha-Amylase in Healthy and Alzheimer's Dementia Brain

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Elin Byman Shatri

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Abstract

Alzheimer's dementia (AD) is the most common form of dementia, with over 40 million diagnoses worldwide, and the number is expected to increase in the coming years. The severe neurodegeneration seen in AD brains is associated with characteristic pathological changes, manifested as amyloid-beta (Aβ) plaques and neurofibrillary tau tangles (NFT), but also as neuroinflammation and impaired brain glucose metabolism. The impaired glucose metabolism occurs early in AD, even before symptoms appear. The loss of sufficient glucose supply is suggested to partly underly the neuronal and synaptic loss associated with the disease. Neuronal signaling is the most energy demanding action in the human brain and is thus in constant need of glucose as an energy source. To secure this need, the brain stores glucose as glycogen. Glycogen is foremost found in astrocytes but also in neurons, where it is used as an energy reserve. The reserve has been shown to be particularly important for memory formation processes such as long-term-potentiation. Hence, dysfunctional glycogen degradation, caused by, for example by AD pathological changes, may cause memory impairments. The brain glycogen is known to be degraded by two enzymes; glycogen phosphorylase and glycogen debranching enzyme, but the human body can produce other glycogen degrading enzymes. The most abundant glycogen degrading enzyme is alpha (α)-amylase, but its presence in the brain and whether it is affected by AD pathological changes has previously not been shown. The aim of this thesis was, therefore, to investigate if α-amylase is endogenously produced in the brain, and if so, explore its functions and roles in AD. By analyzing human post-mortem hippocampal tissue and astrocytic and neuronal cell cultures, we were able to show that α-amylase is expressed and active in the human brain and is found specifically in neuronal dendritic spines and astrocytes. Experimental cell culture studies further suggested that the function of α-amylase in these cells is to degrade glycogen and regulate neuronal signaling. The activity, gene expression, and levels of brain α-amylase were changed in AD patients. These changes appeared to be cell type-dependent, as the amount of α-amylase in neuronal dendrites was reduced, and the number of activated astrocytic containing α-amylase was instead increased. Similar cell dependent impacts on astrocytic and neuronal α-amylase were seen after stimulation with aggregated Aβ. Finally, population-based data was analyzed to investigate the impact of genetic differences in α-amylase production on AD. Interestingly, high copy numbers of the α-amylase gene showed to be associated with lower risk for AD and better episodic memory. In conclusion, these results highlight α-amylase as a possible glycogen degrading enzyme in the human brain and a potential agent in memory formation and AD progression.

Key words

Alzheimer's dementia, Amyloid-beta, Alpha-Amylase, Glycogen, Neurons, Astrocytes, hippocampus

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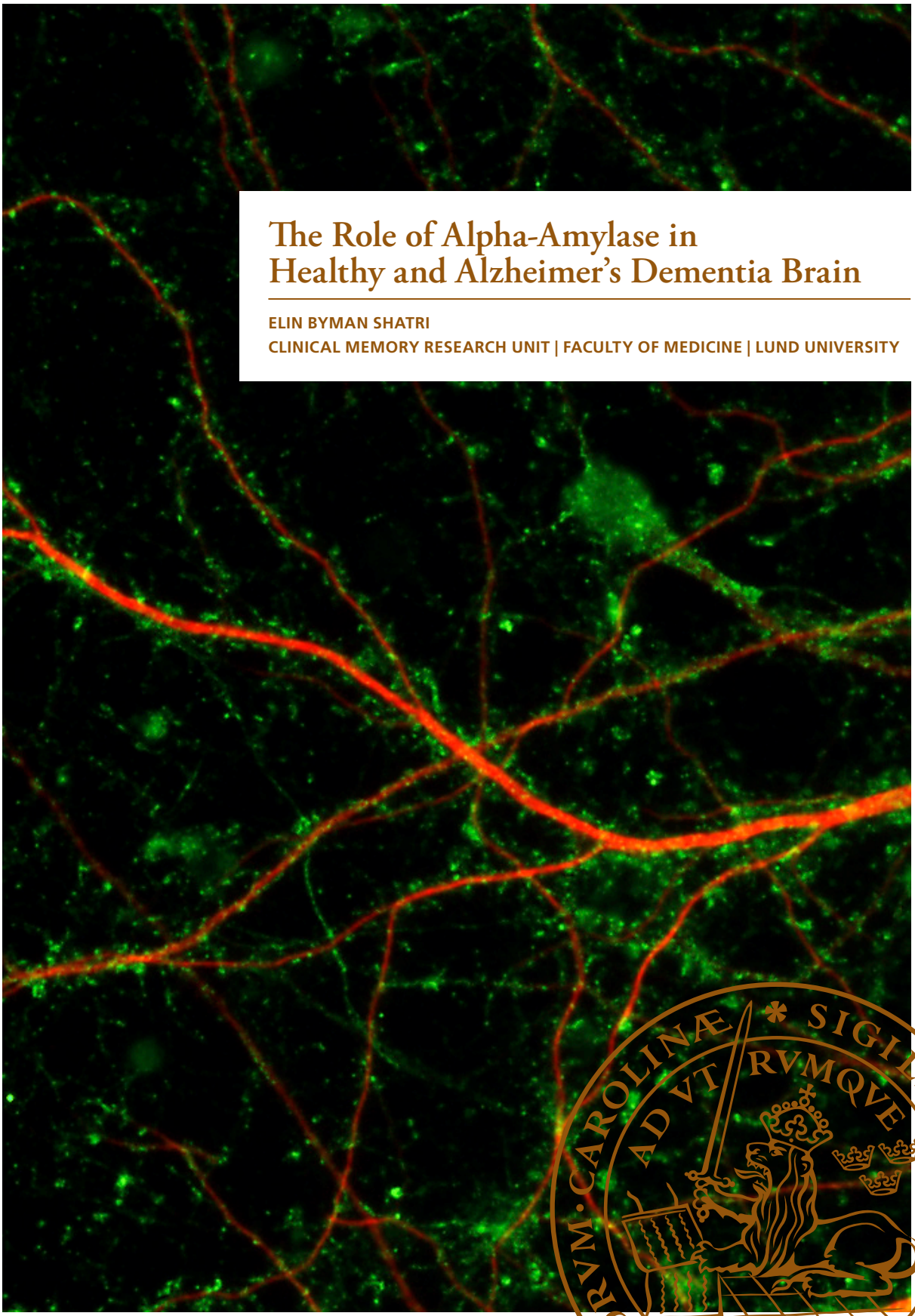

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Cover photo taken by Elin Byman Shatri: *Alpha-amylase (green) in primary mouse neuronal dendrites (red)*

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Abstract

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List of publications

Paper I **Brain alpha-amylase: a novel energy regulator important in Alzheimer's disease?** Elin Byman, Nina Schultz, Netherlands Brain Bank, Malin Fex, Malin Wennström. Brain Pathology, 2018

Paper II **A Potential role for α-amylase in amyloid-β-induced astrocytic glycogenolysis and activation.** Elin Byman, Nina Schultz, the Netherlands Brain Bank, Anna M. Blom and Malin Wennström. Journal of Alzheimer's Disease, 2019

Paper III **Alpha-amylase 1A copy number variants and the association with memory performance and Alzheimer's dementia.** Elin Byman, Katarina Nägga, Anna-Märta Gustavsson, The Netherlands Brain Bank, Johanna Andersson-Assarsson, Oskar Hansson, Emily Sonestedt, and Malin Wennström. Alzheimer's Research and Therapy, 2020

Paper IV **Neuronal α-amylase is important for neuronal activity and glycogenolysis and reduces in presence of amyloid beta pathology.** Elin Byman, Isak Martinsson, Henriette Haukedal, The Netherlands Brain Bank, Gunnar Gouras, Kristine K. Freude and Malin Wennström. Aging Cell, 2021

Abbreviations

α-amylase	Alpha-amylase
Aβ	Amyloid-beta
Aβ 1-40	Amyloid beta 40
Aβ 1-42	Amyloid beta 42
AD	Alzheimer's dementia
APOE	Apolipoprotein E
APP	Amyloid precursor protein
AMPA	α-amino-3-hydroxi-5-metyl-4-isoxazol-propansyra
AMY1A	Alpha-amylase 1A
AMY2A	Alpha-amylase 2A
AMY2B	Alpha-amylase 2B
ANLS	Astrocyte-Neuron-Lactate-Shuttle
BMI	Body Mass Index
CA (1-3)	Cornus Ammonis (1-3)
CAA	Cerebral Amyloid Angiopathy
CAMKII	Ca2+/calmodulin-dependent protein II
cAMP	Cyclic AMP
CERAD	Consortium to Establish a Registry for Alzheimer´s Disease
CN	Copy number
CNV	Copy number variants
CSF	Cerebrospinal fluid
CT	Computerized tomography
CTF-β	Beta-carboxyl-terminal fragment
DE	Debranching Enzyme
DG	Dentate gyrus
cDNA	Coding DNA
EC	Entorhinal cortex
ELISA	Enzyme Linked Immunosorbent Assay
FAD	Familial type of AD
FDG-PET	Fluorodeoxyglucose Positron Emission Tomography
G1P	Glucose-1-phosphate

G6P	Glucose-6-phosphate
GAA	Acid-alpha-glucosidase
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GLUT	Glucose transporters
GP	Glycogen phosphorylase
GPK	Glycogen phosphorylase kinase
GS	Glycogen Synthetase
GSK-3β	Glycogen synthetase kinase-3beta
hiPSC	Human induced pluripotent stem cells
IF	Immunofluorescent
IHC	Immunohistochemical
L150P PSEN1	hiPSC carrying mutation in PSEN1
L150P PSEN1-GC	hiPSC carrying its gene-corrected isogenic control
LOAD	Late onset AD
LTD	Long-term depression
LTP	Long-term potentiation
MAP2	Microtubule Associated Protein 2
MCI	Mild Cognitive Impairment
MDCS	Malmö diet and Cancer Study
MMSE	Mini-Mental State Examination
MoCA	Montreal Cognitive Assessment
mRNA	Messenger RNA
NBB	Netherlands Brain Bank
NC	Non-demented Controls
NFT	Neurofibrillary tangles
NFTL	Neurofilament light chain
NIA	National Institute of Ageing
NMDA	N-metyl-D-aspartat
PAS	Periodic acid Shiff
PFA	Paraformaldehyde
PGB	Polyglucosan bodies
PKA	Protein kinase A
PKM	Pyruvate kinase
PSEN1	Presenilin 1 gene
PSEN2	Presenilin 2 gene
p-tau	Phosphorylated tau

ROS	Reactive oxidative species
RT-qPCR	Quantitative reverse transcriptase polymerase chain reaction
sAPPα	Soluble alpha-APP
sAPPβ	Soluble beta-APP
SDS-PAGE	Sodium-dodecyl-sulphate–polyacrylamide gel electrophoresis
SUB	Subiculum
TEM	Transmission Electron Microscopy
UDP-glucose	Uridine diphosphate glucose
UTP	Nucleotide uridine triphosphate
WT	Wild Type

Populärvetenskaplig Sammanfattning

Alzheimers sjukdom är den vanligaste formen av demens och över 40 miljoner människor beräknas leva med sjukdomen världen över. Den som drabbats av Alzheimers sjukdom uppvisar ofta symptom som minnessvårigheter och begränsningar i tankeförmågan. Dessa symptom är en konsekvens av att hjärnans nervceller dör, vilket sker på grund av sjukdomsspecifika förändringarna i hjärnan. Förändringarna leder till att vissa protein ansamlas och bildar s.k. senila plack och tangles, men även andra förändringar är förknippade med Alzheimers sjukdom. Till exempel ser man ofta att astrocyter, en celltyp som är viktiga för hjärnans immunförsvar och näringsupptag, är aktiverade. Man ser också ett minskat sockerupptag i hjärnan hos Alzheimers patienter. Det minskade sockerupptaget kan börja tidigt i sjukdomsförloppet, till och med innan man ser de första symptomen. Det är viktigt att hjärnan alltid har tillgång till socker, eftersom det är dess främsta energikälla och hjärnans aktivitet kräver mycket energi. Hjärnan lagrar därför socker genom att sätta ihop sockermolekyler till långa förgrenade kedjor, som kallas för glykogen. I en glykogenmolekyl kan flera tusen sockermolekyler förvaras och bidra till en energireserv utifall minskad tillgång på socker skulle uppstå. På senare år har forskning visat att glykogen i hjärnan är extra viktigt för att minnen ska bildas och att problem med att bryta ner glykogen till socker kan leda till minnesstörningar. Problem med nedbrytning av glykogen kan vara kopplat till amyloid-beta, det protein som bildar de senila placken i hjärnan hos patienter med Alzheimers sjukdom.

Man känner till att det finns enzym som bryter ner glykogen i hjärnan, men det enzym som är vanligast förekommande i kroppen, och mest känt för sina egenskaper att bryta ner glykogen effektivt, är ett enzym som kallas alfa-amylas. Man hittar alfa-amylas framför allt i vår saliv och tarm, men enzymet kan tillverkas i mindre mängder i ett flertal olika organ i kroppen. Funktionen av alfa-amylas i munnen och tarmkanalen är främst att bryta ner sockerkedjor (kolhydrater) i den mat som vi äter, men funktionen i andra organ, tex i levern, är troligtvis att bryta ned glykogen för att ge energi till organens celler. Även om det är känt att alfa-amylas tillverkas och finns i många organ, har det fram till nu inte funnits några rapporter om att alfa-amylas finns och är aktivt i hjärnan. Syftet med studierna i denna avhandling var därför att undersöka om alfa-amylas tillverkas i hjärnan, och vilken funktion det isåfall har. Syftet var också att undersöka om uttrycket av alfa-amylas påverkas vid

eller kan bidra till Alzheimers sjukdom. För att ta reda på detta undersökte vi närvaron av alfa-amylas i hjärnvävnad från avlidna individer med och utan Alzheimers sjukdom och i odlade astrocyter och nervceller. Resultaten visade att alfa-amylas tillverkas i hjärnan och finns specifikt i både astrocyter och neuron. Våra försök på odlade celler visade också att enzymet troligen bryter ner glykogen i dessa celler. I astrocytens fall verkade alfa-amylasnedbrytningen av glykogen vara kopplat till aktivering av cellen, medan den i nervcellens fall visade sig vara viktigt för signalering. Det var därför intressant att vi även såg att uttrycket av det neurala alfa-amylaset var sänkt hos patienter med Alzheimers sjukdom medans alfa-amylas i astrocyter var ökat jämfört med icke-dementa kontroller. Dessutom kunde vi visa att amyloid-beta leder till minskat uttryck av alfa-amylas i odlade neuron och ökat uttryck och aktivitet i odlade astrocyter. Det verkar alltså som om alfa-amylas kan spela en viktig roll vid den astrocyt-aktivering och förlust av nervcellssignalering som man ser vid Alzheimers sjukdom. Slutligen gjordes även en populationsstudie där vi undersökte om genetiska förutsättningar att tillverka alfa-amylas påverkar risken att utveckla Alzheimers sjukdom. Studierna visade att individer som har väldigt många genkopior av alfa-amylas (och därmed högre alfa-amylasproduktion) har både minskad risk för att få Alzheimers sjukdom och ett bättre episodiskt minne. Sammantaget visar dessa studier att alfa-amylas finns i hjärnan och där har en funktion att bryta ner glykogen till energi för driva viktiga processer såsom astrocytaktivering och nervcellsignalering. Vid Alzheimers sjukdom rubbas dessa processer genom att amyloid beta direkt påverkar uttrycket av alfa-amylas. Studierna bidrar därför med ny kunskap om hur hjärnan påverkas vid Alzheimers sjukdom och föreslår alfa-amylas såsom en ny viktig aktör vid processer viktiga för hjärnans funktioner.

Introduction

There are around 86 billion neurons and at least as many non-neuronal cells, intertwined but still perfectly ordered, in the human brain (1). This high density of neurons, and the extraordinarily large prefrontal cortex, are thought to underlie the high cognitive and verbal abilities which characterize the human mind. Our brain is three times larger in terms of brain/body mass index compared to other primates, which is believed to lie behind its exceptional capability. However, this capability may exist beyond genetics. At birth our brains are only a third of their fully grown size, compared with the 70% other primates are born with. This means that the human brain primarily develops postnatally and is therefore highly adaptable to our environment, and social and cultural contexts. The human brain is also extremely ductile with a great ability to change its neuronal structure and function based on internal or external stimuli (2). This ability, called neuronal plasticity, is crucial when memories are formed. The sensory input is converted into chemical signals, which are processes, into a memory based on new neuronal connections. Such synaptic connections are constantly formed but also deleted, leaving our brain unlimited but also extremely vulnerable. Neuronal plasticity decreases with age, resulting in age-related cognition and memory decline. This decline is natural but can accelerate inexorably when pathological changes occur, leading to severe loss of neurons and memory impairment.

Alzheimer's dementia

In 1906 the German psychiatrist Alois Alzheimer described, for the first time, the presence of dense inclusion bodies, later called senile plaques and fibrillar tau tangles (NFT), in the brain of an individual exhibiting confusion, anxiety, and memory impairment (3). The disease he described is what we now call Alzheimer's dementia (AD). This disorder is the most common form of dementia, a group of neurocognitive disorders affecting over 43.8 million people (2016), and the fifth leading cause of death worldwide (4). The prevalence of AD has been predicted to rise to 131.5 million people in 2050, with the highest increase occurring in low- and middle-income countries.

Symptoms

Symptoms of AD develop gradually, frequently beginning with disturbance of the episodic memory. The memory decline is often accompanied by anxiety and depression. As the disorder progresses, additional symptoms arise such as difficulties remembering names or following a conversation, which sometimes leads to withdrawal from social activities. Loss of orientation and behavioral disturbances are also symptoms associated with AD (5).

The mild symptoms seen in the very earliest stages are called Mild Cognitive Impairment (MCI). However, many different conditions can cause MCI and cognitive impairments including normal aging, vitamin deficiency, infections, abnormal reaction to drugs, alcohol abuse, brain tumor, or other neurological disorders (6). Therefore, it is crucial for a patient with MCI to get a thorough evaluation to reach a correct diagnosis.

Diagnostics

Individuals who experience cognitive changes are mostly dealt with at the primary care level where a first assessment is made of medical history, physiological and psychological status. The assessment includes cognitive tests which evaluate various memory and other cognitive functions. The most frequently used test is the *mini-mental state examination (MMSE)*. This comprises a broad evaluation of orientation, memory, language, and logistic-spatial abilities. It takes about 15 minutes to complete and the maximum possible score is 30 points. MMSE is also used to evaluate the degree of dementia, where mild dementia scores 20 and over, moderate 10-19 points, and severe dementia nine and under (7). MMSE is often used together with the *clock test*, which measures constructive abilities, time perception, and reduced planning abilities. This test involves only a pen and a paper and the patient is asked to draw a clock with numbers and draw the hands at a

specific time (8). The *Montreal Cognitive Assessment (MoCA)* test is also commonly used for evaluation of cognitive dysfunction. The test assesses global cognitive function using ten sub-tests; visuospatial, short-term memory, executive functions, phonetic fluency task, two-item verbal abstraction task, attention, concentration, working memory, language, and orientation (9). If a cognitive disorder is suspected, a computerized tomography (CT) of the brain is also performed.

If further evaluation is needed for the diagnosis, the individual can be referred to a specialist clinic for more measures such as lumbar puncture or magnetic resonance imaging (MRI) of the brain. Lumbar puncture collects cerebrospinal fluid (CSF) that can be analyzed to reveal the presence of AD-specific biomarkers reflecting the neuropathological hallmarks of AD i.e., Amyloid beta (Aβ) plaques and NFT. It is also possible to evaluate structural brain changes using technical methods, such as fluorodeoxyglucose positron emission tomography (FDG-PET) measuring the uptake of glucose in the brain (10, 11). The results from all these tests and evaluations form the basis for the clinical diagnosis.

Risk factors

The risk of developing AD increases with age and the sporadic form, called late onset AD (LOAD), is found primarily in individuals over 65 years of age. The prevalence of AD in Europe at 65 years of age is 4.4%, which is doubled every 5th year of life. Among the very old, it is estimated that as many as a third of those over 85 years of age (12) have AD. Apart from aging, several other factors are associated with AD. Lifestyle factors such as smoking have, for example, been shown to significantly increase the risk of AD (13). Lower education level has also been identified as a risk factor together with cardiovascular diseases and type 2 diabetes (14-16). In addition, several genes related to Amyloid Precursor Protein (APP) processing (*ADAM10*), neuroinflammation (*TREM2*, significant but rare), immunity (*HLA-DR, CR1*) and lipid transportation (*CLU*), to mention a few, have been associated with LOAD (17). The most significant genetic risk factor for LOAD (to our knowledge) is polymorphism in apolipoprotein E (*APOE*), which is represented by three alleles; ε2, ε3 and ε4. Only ε4 is associated with an increased risk of developing AD, where ε4 heterozygotes present a 3-4-fold increased risk and ε4 homozygotes up to a 15-fold increased risk (18, 19).

The familial type of AD (FAD), accounts for about 1-2% of all AD cases and often debuts before 65 years of age. FAD is caused by mutations in genes that transmit through autosomal dominant inheritance (20). These mutations are found in proteins that are involved in the formation of senile plaques, *amyloid precursor protein gene* (*APP*), the *presenilin 1 gene* (*PSEN1*), and the *presenilin 2 gene* (*PSEN2*), with more than 300 different gene mutations reported (19).

Cognitive reserve

In contrast to the risk factors, there are several factors associated instead with delayed onset of MCI and reduced risk of AD (21). Higher education, higher IQ, social and physical activities, a healthy diet, lifelong mental learning, and bilingualism are factors thought to enhance resilience to AD by creating a cognitive reserve (22-26). The term cognitive reserve implies that individuals have different cognitive resources, and the symptoms of aging or AD become apparent when these resources fall below a certain threshold (27) (Figure 1). Studies have shown that engaging in activities, even early in life, leads to reduced risk of AD. To achieve a full effect regarding resilience and reduced risk of AD, the engagements have to be lifelong, leading to an accumulation of cognitive reserve (21, 28). Reduced risk can also be linked to genetic differences where a higher IQ and better memory lead to a greater cognitive reserve (29). Taking all this together, the cognitive reserve is dependent on a combination of genetics, lifestyle and challenges throughout life (27).

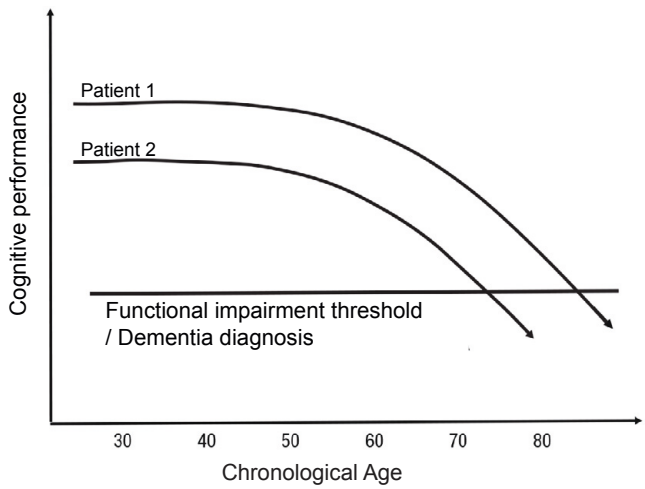

Figure 1. Cognitive reserve. Schematic description of two individuals who gain different cognitive abilities up to the age of 20, and therafter encounter similar life challenges leading to a cognitive decline towards the threshold for functional impairment or dementia. The image is modified from Lövdén et al. *PSPI*, 2020, published under CC BY 4.0 Licence https://creativecommons.org/licenses/by/4.0

Drugs and therapies

AD is a fatal disease and the treatments available can only stabilize its symptoms. Current therapies target two different molecular mechanisms; degradation of acetylcholine by inhibiting acetylcholinesterase (*Donepezil*, *Galantamin* and *Rivastigmin*) and inhibition of the N-Methyl-d-Aspartate (NMDA) receptor using an antagonist (*Memantine*). The acetylcholinesterase inhibitor increases signaling in neuronal synapses and is efficient mainly in the early stages of the disease,

prolonging the autonomy and life expectancy of patients (30, 31). The NMDA antagonist blocks the effects of pathologically high levels of glutamate and thereby enhances cognitive functions and reduces behavioral disturbances (32, 33).

In recent years, much effort has been invested in finding a way to remove the Aβ-plaques and NFTs from the brain. Although several immuno-therapies, directed against Aβ-plaques, have progressed as far as clinical trials all have failed for various reasons. The exception is *Aducanumab,* which was recently approved by the Food and Drug Administration in the USA, as a treatment for AD (34). Europe and Sweden have not approved it since the effect of the drug remains uncertain.

Alzheimer's disease pathology

The cognitive impairments seen in AD are linked to the atrophy of brain areas, especially in the hippocampus, the memory processing centre in the brain. The atrophy is due to loss of neurons and synapses, so-called neurodegeneration, which is thought to be caused by specific pathological changes. As mentioned above, the pathological hallmarks of AD are the presence of Aβ-plaques and Neurofibrillary tau tangles (NFT) (35, 36). However, other pathological changes such as neuroinflammation, manifested by activation of glial cells, and altered glucose hypometabolism are seen in AD patients (37-40). These pathological changes are thought to start several years before the onset of AD symptoms, which have been shown by studies investigating changes in CSF biomarkers (Aβ, p-tau, and neurofilament light chain), as well as FDG-PET (glucose metabolism) and amyloid-PET (Aβ burden) (36, 39, 41). Interestingly, a study on individuals with FAD (i.e., individuals carrying mutations leading to an increase in Aβ production) showed that Aβ accumulation starts 22 years before the expected onset of AD. Hypometabolism starts 18 years and atrophy 13 years before the expected onset of AD (42). Hence, much evidence points towards Aβ accumulation being the initiating pathological step in AD. This belief has however been debated, mostly due the failure of anti-amyloid therapies in recent years, but also since some studies suggest other mechanisms (for example tau phosphorylation, neuroinflammation, glucose metabolism) as alternative initiators (43-46).

Neuropathological evaluations

NFT and Aβ-plaques are found at the beginning of the disease in specific brain regions and spread in a predictable pattern throughout the brain as the disease progresses. This spreading has been described by Braak and Braak, who also defined a staging system, published in 1991 (47). The staging system is divided into six different stages for NFT spreading (I-VI) and three for Aβ-plaque density (A-C);

NFTs are initially found in the peripheral regions of the entorhinal cortex (EC) and hippocampus (I) and then spread along the EC and CA1 region of the hippocampus (II). In stage III, NFT accumulates in the subiculum and in stage IV in the amygdala, thalamus, and claustrum. In stage V, the NFT spreads to the isocortical areas, and finally, in stage VI, the primary sensory, motor and visual areas are affected (47). The spread of Aβ is not as entirely predictable as that of NFT. In stage A, accumulation of Aβ is seen in the basal portions of the isocortex, in stage B it is present in isocortical areas and mildly in the hippocampus, and finally, in stage C, a higher density of Aβ depositions can be seen throughout the isocortex, including the sensory and motor cortex (47).

Additional protocols for neuropathological assessment systems of NFTs and Aβ plaques have since been published. One example of such a staging system is CERAD, which is a semiquantitative scoring of neuritic plaques based on algorithms and the neuropathologist's opinion, which evaluate whether the AD diagnosis is 1=Definite, 2=Probable, 3=Possible and 4=No AD (48, 49). The use of the Braak and Braak NFT staging system in combination with CERAD was recommended by the consensus report (NIA) published in 1997 (50).

Amyloid beta cleavage and aggregation

The AD characteristic Aβ plaques are thought to appear due to an imbalance between production and clearance of the Aβ peptide (51). The peptide is produced by the cleavage of amyloid precursor protein (APP). APP is an integral membrane protein highly expressed in neurons but can also be expressed in other brain and peripheral cells. The protein is cleaved along two different cleavage pathways; the non-amyloidogenic and the amyloidogenic. In the non-amyloidogenic pathway, APP is cleaved by α-secretase forming the soluble alpha-APP (sAPPα), which is involved in neuronal plasticity (52). In the amyloidogenic pathway APP is instead cleaved by β-secretase yielding soluble beta-APP (sAPPβ). The remaining c terminal fragment C99 (or CTF-β) in the membrane is further cleaved by γ-secretase (where a subpart of the enzyme is presenilin) into Aβ (Figure 2) (53). Depending on the cleavage site of γ-secretase, fragments of different lengths are produced (43, 45, 46, 48, 49, and 51 amino acids). These fragments are further processed into the main Aβ forms, Aβ1-40 or Aβ1-42 amino acids.

The monomeric form of Aβ has been shown to have important physiological roles. For example, Aβ1-42 monomers can be neuroprotective (54) and promote glucose uptake in neurons (55). *In vitro* studies on human brain pericytes show that Aβ1-40 monomers lower caspase 3/7 activity and are beneficial for cell proliferation (56). However, Aβ monomers can also aggregate and form oligomers, ranging from low-molecular-weight, including dimers, trimers, tetramers, and pentamers, to mid-range molecular weight oligomers, including hexamers, nonamers, and dodecamers,

which are all soluble. Aβ can further aggregate into protofibrils, which are also soluble, and fibrils, which are insoluble (19, 57). All aggregation forms are reversible, where monomers can be added or removed. Although, Aβ fibrils are considered to be the main component in Aβ plaques, it has been shown that oligomers and protofibrils are the most cytotoxic forms of Aβ (58). Aggregates of Aβ1-40 are found particularly in small and medium-sized arteries; a condition called Cerebral Amyloid Angiopathy (CAA), causing cerebral haemorrhage (59).

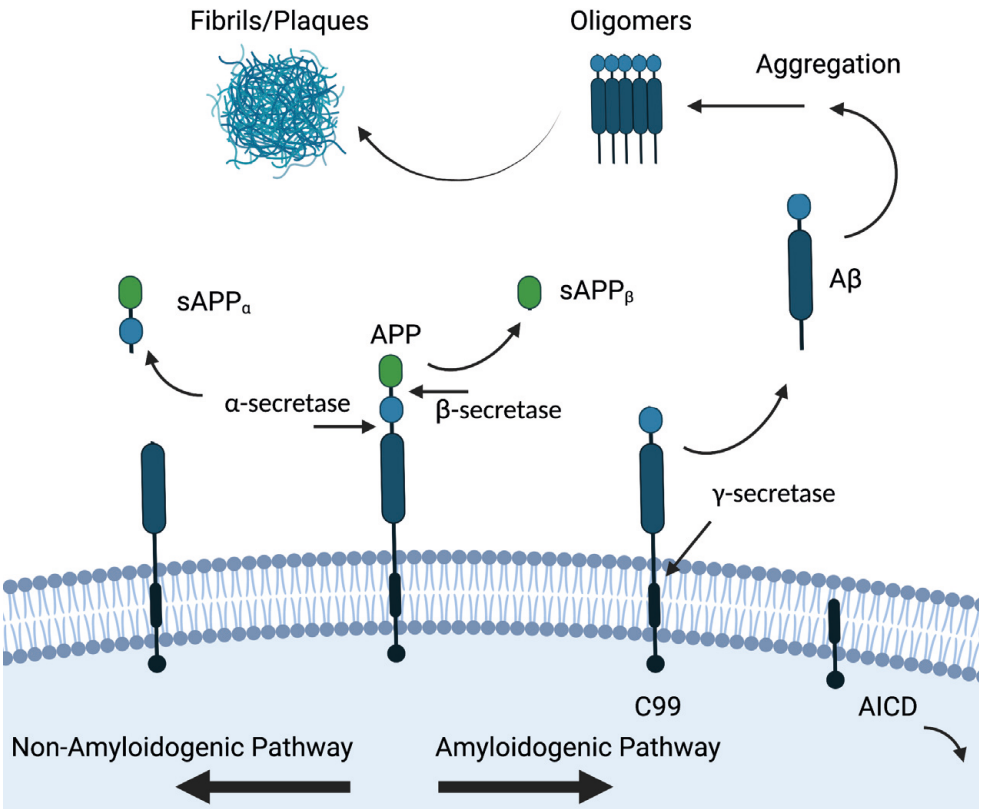

Figure 2. Amyloid Precursor Protein (APP) cleavage and Amyloid beta (Aβ) aggregation. The non-Amyloidogenic pathway is initiated by the cleavage of APP by α-secretase resulting in the release of soluble APPalpha (sAPPα). The Amyloidogenic Pathway is initiated by the cleavage of APP by β-secretase, which results in the release of soluble APPbeta (sAPPβ) and leaves the C99 fragments in the membrane. The C99 fragments are thereafter cleaved by γ-secretase, which results in the production of Aβ1-40 or Aβ1-42 monomers, the AICD fragment is internalized into the cell. In pathological conditions, the monomeric forms of Aβ1-40 or Aβ1-42 can start to aggregate and form oligomers. These oligomers are further aggregated into Aβ fibrils and Aβ plaques. Aβ plaques can be seen in brain of individuals with Alzheimer's dementia and are a neuropathological hallmark for the disease. Created with BioRender.com

AD Neurofibrillary tau tangles

NFTs and neuropil threads (NTs) can be found in several different neurological disorders (including Parkinson's disease and Frontotemporal dementia) apart from AD, and even in elderly normally cognitive individuals (60, 61). The formation of the pathological structure is linked to hyperphosphorylation of tau (62), a microtubule binding protein promoting stability in the neuron. The hyperphosphorylation dissociates tau from the microtubule and initiates an assembly of phosphorylated tau (p-tau) into oligomers and fibrils (63). Neurons containing NFTs display a disrupted cytoskeleton, which affects both the stability and transportation of vesicles along the axons (64).

The consensus in the AD field is that Aβ accumulation is an upstream event of tau phosphorylation in AD pathogenesis (10). Aβ can induce phosphorylation of tau (65), which might be mediated by reactive oxidative species (ROS), leading to the activation of kinases, which further hyperphosphorylates tau (66, 67). Aβ can also bind to membrane receptors causing a signaling cascade leading to activation of kinases that further phosphorylates tau (68-72). One of these kinases is Glycogen synthetase kinase 3beta (GSK-3β), which phosphorylates tau at multiple AD-specific sites (73-77) and has therefore been proposed as an important link between Aβ and tau-phosphorylation (78, 79).

Inflammation

A common pathological feature of AD is neuroinflammation, characterized by the presence of reactive microglia and astrocytes. These glial cells are part of the innate immune system in the brain and have important functions such as detecting and phagocytosing pathological substances. Aggregated Aβ, is one of these substances. When patrolling glial cells encounter Aβ, they become activated via binding of Aβ to toll-like receptors at the surface of the glial cell (80). The activation is manifested by a change in morphology and increased production and secretion of cytokines, leading to the recruitment of more glial cells (81-83). These activated astrocytes and microglia are often seen in the vicinity of Aβ plaques, where they form a protective barrier and/or clear the plaques by phagocytosis (84-86). However, in AD the glial cells are dysfunctional (80), initiate an abnormal synapse pruning and promote neurodegeneration (87, 88).

Glucose metabolism

Impairment in glucose metabolism is one of the earliest pathological changes seen in AD and can be visible several years before symptoms appear (39, 89) (90, 91). The reduction of glucose metabolism in the brain is considered to be a consequence of the severe neurodegeneration in AD, where the loss of neurons and synapses

reduces the demand for glucose. Since glucose is the main energy source in the brain, disruption in its metabolism can have fatal consequences for brain cells, particularly neurons and synapses (92). Additionally, metabolic diseases such as type 2 diabetes increase the risk of developing AD (16, 93, 94) and, interestingly, AD patients show brain insulin resistance (95-97), due to downregulation of hippocampal insulin receptors and insulin-like growth factor (IGF-1). This, together with the findings that glucose transporters and key glycolytic enzymes are reduced in the early stages of AD, might indicate that AD hypometabolism is connected to other pathological changes besides loss of neurons (98).

Memory formation

Formation of memories, in particular the consolidation of short-term memory into long term memory, is dependent on a structure called the hippocampus. This structure is embedded deep in the temporal lobe and is shaped like two interlocked C's, resembling the form of a seahorse (hence the name hippocampus; the Latin word for seahorse). The hippocampal formation comprises the hippocampal proper with Cornus Ammonis (CA) 1-3, the dentate gyrus (DG), the subiculum (SUB), and EC (Figure 3) (99). The hippocampus and DG are further organized in three layers. The three layers of the hippocampus are the polymorphic layer, the pyramidal layer, and the molecular layer and the layers of dentate gyrus are the polymorphic layer, granular layer, and molecular layer. Neurons in the hippocampus can be found in different regions and layers. The major neuronal type in the hippocampus is the pyramidal neurons, found primarily in CA1 and CA3. Pyramidal neurons have a triangle-shaped cell body localized and arranged in the pyramidal layers. They have a thick apical dendrite that goes through the molecular layer and several basal dendrites that go through the polymorphic layer. These basal dendrites can be up to 200-300 μm long, and just like the apical dendrite, they are covered with dendritic spines. Other neuronal types in the hippocampus are granular cells including, mossy fibres, found in DG, and a variety of different types of interneurons.

The hippocampus receives high levels of sensory information from several different brain regions which enters via the EC. This pathway is called the perforant pathway, which inputs signals through the neuronal circuits in the hippocampus from EC via DG to CA3 and further to CA1 and SUB, and the output signaling goes back to EC (Figure 3) (100).

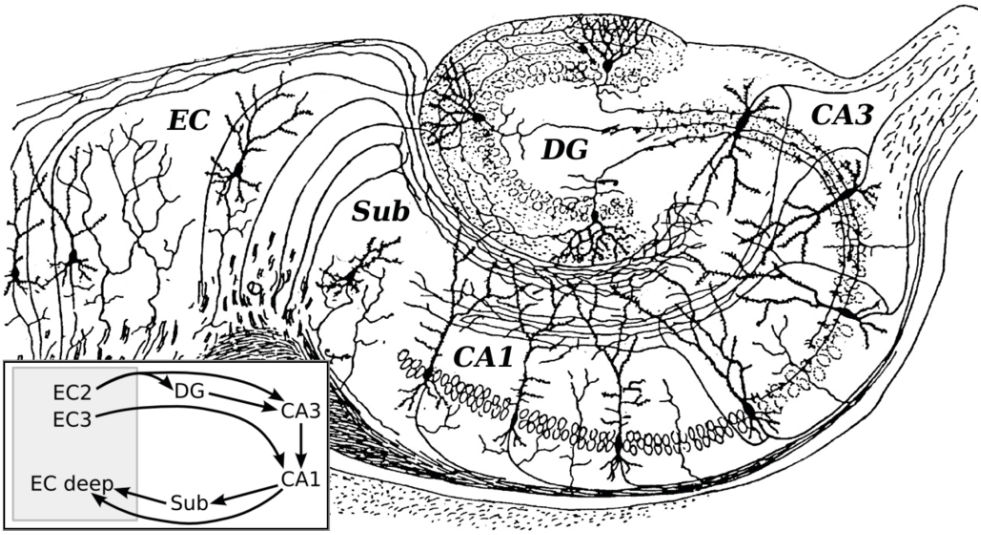

Figure 3. Illustration of rat hippocampus. The illustration shows the different regions of the hippocampal formation with Dentate gyrus (DG), Cornius Ammonis 3 (CA3), Cornius ammonis 1 (CA1), Subiculim (Sub) and Enthorinal cortex (EC). The box in the left corner shows the major neuronal circuit in the hippocampus with sensory imputs from EC further to DG, CA3 or CA1 and via Sub is signaled back to EC. (Modified from Cajal 1911)

The mechanism involved in memory formation is not fully understood, however, the formation of long-term potentiation (LTP) is thought to be key. The LTP is formed when a signaling sequence, which last for several minutes up to hours, resulting in an upregulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and increased glutamatergic synaptic sensitivity (101). The LTP can further be modulated by locus coeruleus derived norepinephrine, which acts upon β-adrenergic receptors. This signaling cascade is further boosting AMPA mRNA translation and thereby memory endurance (102, 103). This makes the synapse is more sensitive towards glutamate and thus more likely to be activated again.

Glycogen

The ability to form memories comes at a high price, as LTP formation, neuronal plasticity and synaptic activity require a large amount of energy. Synaptic activity alone is the most energy-demanding action within the human body and accounts for 75% of the brain's total energy use, while the brain itself uses 20% of the body's total energy use (104). Hence, the brain needs a constant supply of energy. The main energy source is glucose, which is transported from the periphery into the brain through the blood. The brain also has the ability to store glucose in larger sugar molecules called glycogen, which then is used to meet a high energy demand or low

glucose supply. The storage of glucose in the form of glycogen occurs throughout the human body and is considered to be the major mammalian form of carbohydrate storage. The largest quantity can be found in the liver, constituting approximately 8% of its total weight and playing an essential role in maintaining normal blood glucose levels. The skeletal muscles contain 1% glycogen (105), used primarily as an energy substrate for muscular toning and aerobic training. However, lactate, a rest produced when glycogen is degraded in anaerobic conditions via glycolysis, can be released from the skeletal muscles into the blood and serve as an energy substrate for other cells (106). The discovery of this event, called the "Cori cycle", led to a Nobel Prize award in 1947. The brain is estimated to contain only 0.1% glycogen and thus has long been neglected in research, as the concentration is too low to compensate for a disrupted glucose supply (105, 107). However, we know today that brain glycogen can not only support brain cells with energy for shorter periods, but that it also has a dynamic role, where it is continuously degraded and synthesized for several purposes (108-110).

Function of glycogen in the brain

A functional role for glycogen in the brain was first proposed in 1992 by Swanson et al. They were able to demonstrate utilization of glycogen in astrocytes when rat face and vibrissae were stimulated (111), indicating a connection between glycogen degradation and neuronal activity. Several follow-up studies have shown that glycogen is also important in learning and memory formation. Gibbs et al. demonstrated that both inhibiting glycogen degradation and decreasing glycogen-dependant glutamate production cause impairment of learning and memory in chickens (112, 113). Further, in 2011 Suzuki et al. confirmed the connection between glycogen and memory, specifically the formation of LTP. They showed that an inhibiting of a glycogen degrading enzyme in the rat hippocampus impaired LTP formation, an effect that could be remedied by an injection of lactate (114). This finding suggests that LTP is dependent on lactate produced from glycogen degradation (114). Additionally, transgenic mice lacking glycogen synthetase have been shown to exhibit impairment of memory formation (115) and recently, formation in LTP was shown in mice specifically lacking neuronal glycogen (116); again supporting the idea that glycogen plays a crucial role in memory formation.

The Astrocyte-Neuron-Lactate-Shuttle hypothesis

While neurons have the highest energy demand (104), it is well-established that astrocytes metabolize glucose to a much higher extent and is the main producer of brain glycogen. This knowledge has led to the assumption that astrocytes provide neurons with energy retrieved from its glycogen storage. But since G6P (the end-

product of GP glycogen degradation) cannot be transported over the glial and neuronal membrane, G6P needs to be converted into something that can. Glucose is transported via the GLUT transporters, but since astrocytes lack glucose-6-phosphatase, they are unable to convert G6P into free glucose (117). They can however convert G6P into lactate. This knowledge is one of the corner stones in a hypothesis called "Astrocyte-Neuron-Lactate-Shuttle" (ANLS) (118). The hypothesis proposes the following scenario. When glutamatergic neurons are firing, the surrounding astrocytes take up the released glutamate. In turn, glutamate activates glycogen degradation and the downstream glycolysis. After conversion of pyruvate into lactate, where the latter is shuttled to neurons (Figure 4). Meanwhile, astrocytes convert the glutamate into glutamine and release it back to neurons. Neurons convert the glutamine into either to glutamate or gamma-aminobutyric acid (GABA) (119). Interestingly, astrocytes can also provide *de novo* synthesis of glutamate and glutamine from an intermediate product of the TCA cycle, α-ketoglutarate; a process shown to be dependent on glycogen degradation (117).

Although several studies, including findings of elevated lactate levels in the brain during neuronal activation (120-122), support the ANLS hypothesis, the hypothesis been questioned. Some argue that there is no *in vivo* evidence for a transportation of lactate from astrocytes to neurons to occur (123) and studies supporting the ANLS hypothesis do not add up in stoichiometry (124). Additionally, studies have shown that neurons are fully capable of taking up glucose and supporting their own energy needs (125-127) and therefore there is no need for any astrocytic-derived lactate.

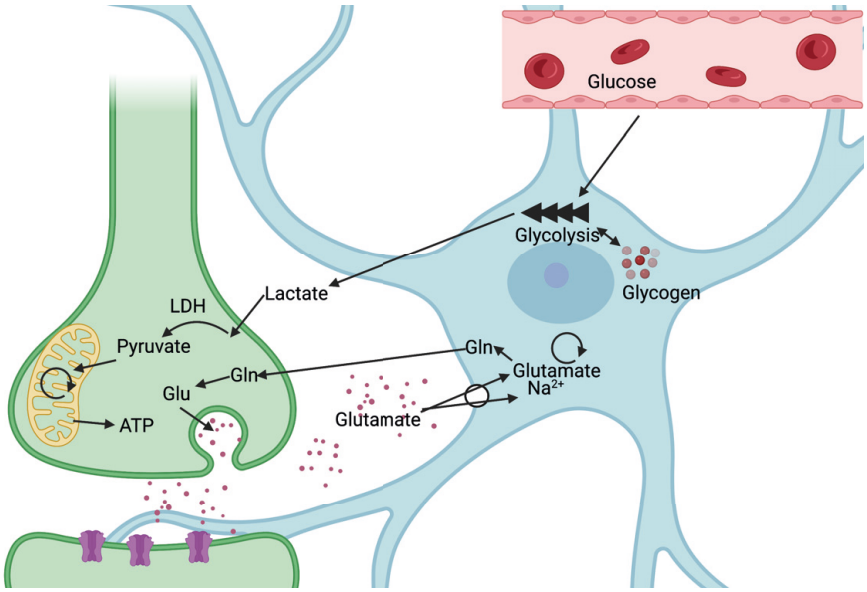

Figure 4. Illustration of the Astrocyte-Neuron-Lactate-Shuttle. Glutamate (Glu) is released from glutamatergic neurons, taken up by nearby astrocytes and further converted into glutamine. The glutamine is then released to the extracellular space, where it is taken up by neurons to be reused as glutamate again. This Glutamate-Glutamine cycle activates the TCA cycle and glycolysis. Glycogen is degraded by glycogen phosphorylase into glucose-6-phosphate, which enters the glycolysis with the end product pyruvate. Pyruvate needs to be further converted into lactate in order to be released into the extracellular space. The released lactate is taken up by nearby neurons, converting it to pyruvate with lactate dehydrogenase (LDH). The pyruvate can now enter the TCA cycle and produce ATP for the neuron. Created with BioRender.com

Glycogen synthesis

The tree-like structure that characterizes the glycogen molecule, was proposed as early as 1940 by Meyer and Bernfield, and later on the composition of highly branched glucose molecules connected with α1-4 and α1-6 glycosidic bonds was defined (128). A single glycogen molecule can harbour up to 55,000 glucose units in a water-soluble form. This makes glycogen a perfect molecule which store large amounts of glucose at low osmotic pressure (129). The storage and metabolism of glycogen is primarily located in astrocytes, but neurons can also produce and degrade glycogen, although at lower levels (107, 116, 129-132).

The synthesis of glycogen in astrocytes starts with the active uptake of periphery-derived glucose via astrocytic end-feet, covering up to 60-90% of the vasculature in the brain (133, 134). The uptake is regulated by glucose transporters (GLUT), which can be found in both astrocytes (GLUT1) and neurons (GLUT3) (135). Immediately after uptake, the glucose is converted into glucose-6-phosphate (G6P) by the enzyme hexokinase. There are three possible trajectories for G6P: i) entering the glycolysis and further into the TCA cycle; ii) entering the Pentos phosphate pathway; or iii) be synthesized into glycogen (136). When entering the glycogen synthesis, G6P is

catalysed by the enzyme phosphoglucomutase, which transfers a phosphate group from carbon 6 to carbon 1, resulting in the formation of glucose-1-phosphate (G1P). The G1P then reacts with nucleotide uridine triphosphate (UTP) to form the activated glucose form uridine diphosphate glucose (UDP-glucose). The UDP-glucose is then added to the protein glycogenin to form glycosylglycogenin, which acts as a primer for glycogen synthesis. The molecule grows with additional UDP-glucose molecules added with α1-4 glycosidic bonds, a reaction catalysed by the enzyme glycogen synthetase (GS). The branches of glycogen with α1-6 glycosidic bonds are subsequently formed by the branching enzyme (Figure 5) (137, 138).

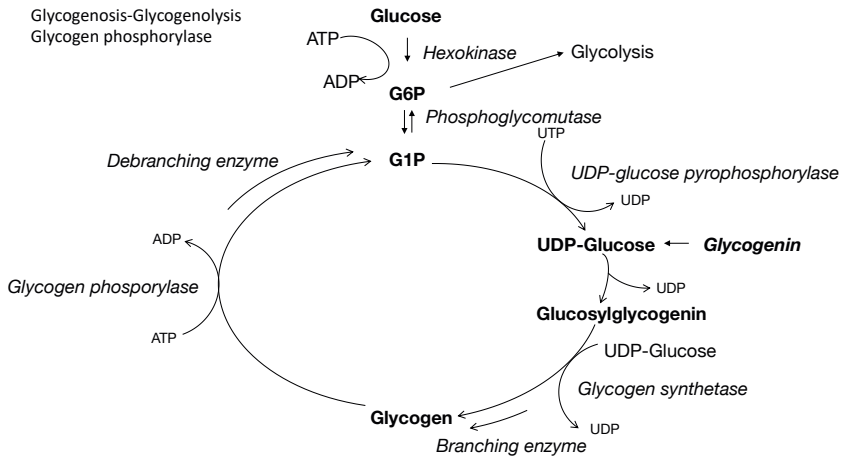

Figure 5. Glycogenosis; Schematic image of glycogen metabolism in brain involving the enzymes Glycogen Synthetase and Glycogen Phosphorylase. Glucose is taken up from the blood and immediatly converted into glucose-6-phosphate (G6P). The G6P can have three possible trajectories, entering the glycolysis, entering the pentos-phosphate pathway, or being converted into glycogen. For the glycogenosis pathway, G6P is converted into glucose-1-phosphate (G1P) where an UDP molecule is added, forming UDP-glucose. By the binding of UDP-glucose to glycogenin, Glucosylglycogenin is formed, which is a primer for the glycogen molecule. By the action of Glycogen synthetase, additional UDP-glucose molecules are added, and together with branching enzyme, the glycogen molecule is formed. **Glycogenolysis;** The enzyme Glycogen phosphorylase phosphorylates the end glucose molecules of glycogen into G1P, which is further converted into G6P. The Debranching enzyme is needed to cleave of the α1-6 glycosidic bonds, resulting in more end-molecules which can be phosphorylated by Glycogen phosphorylase.

Glycogen synthesis can be regulated in several ways. For example, the amount of glycogen that can be synthesized is determined by the level of glucose uptake, the presence of GLUT and the enzyme hexokinase (139, 140). Additionally, the major regulator of GS is G6P, which induces a conformational change within the enzyme resulting in a higher affinity for the substrate. Phosphorylation of GS, however, leads instead to a lockdown and inactivation of GS (141). Such phosphorylation can occur at different phosphorylation sites and several enzymes responsible for the

phosphorylation of GS have been identified, including glycogen synthetase kinase 3-beta (GSK3β), Protein kinase A (PKA), and calmodulin-dependant protein kinase II (CAMII) (142).

Glycogen degradation

The degradation of brain glycogen is mainly carried out by glycogen phosphorylase (GP). This enzyme exists in both an activated and inactivated form that is regulated by glycogen phosphorylase kinase (GPK). Activated GP causes phosphoroclastic cleavage of the end molecules on glycogen, resulting in the production of G1P (Figure 5). GP can only cleave glucose molecules up to four molecules from a branching point. Therefore, an additional enzyme, called debranching enzyme (DE), is needed to remove the branches so that GP can continue the glycogen degradation. The cleavage product G1P is further converted into G6P, which can be used directly in glycolysis (143). Glycogen can also be degraded in lysosomes by the enzyme acid alpha glycosidase (GAA), this cleavage is done by hydrolysis of the α1-4 glycosidic bond within the glycogen molecules with glucose and maltose as its end products (144).

The signaling cascade leading to the GP activation can be initiated *via* different pathways, where one involves the binding of epinephrine to β-adrenergic receptors and subsequent activation of adenylate cyclase. This results in an elevation of cyclic adenosine monophosphate (cAMP) (145), which in turn activates Protein Kinase A (PKA), initiating a phosphorylation cascade with the activation of GPK and GP as downstream events. Regulation of GP can also be initiated by the allosteric actions of AMP, ATP and G6P, where GP enzymatic activity is accelerated by AMP and it is slowed down by ATP and G6P (Figure 6).

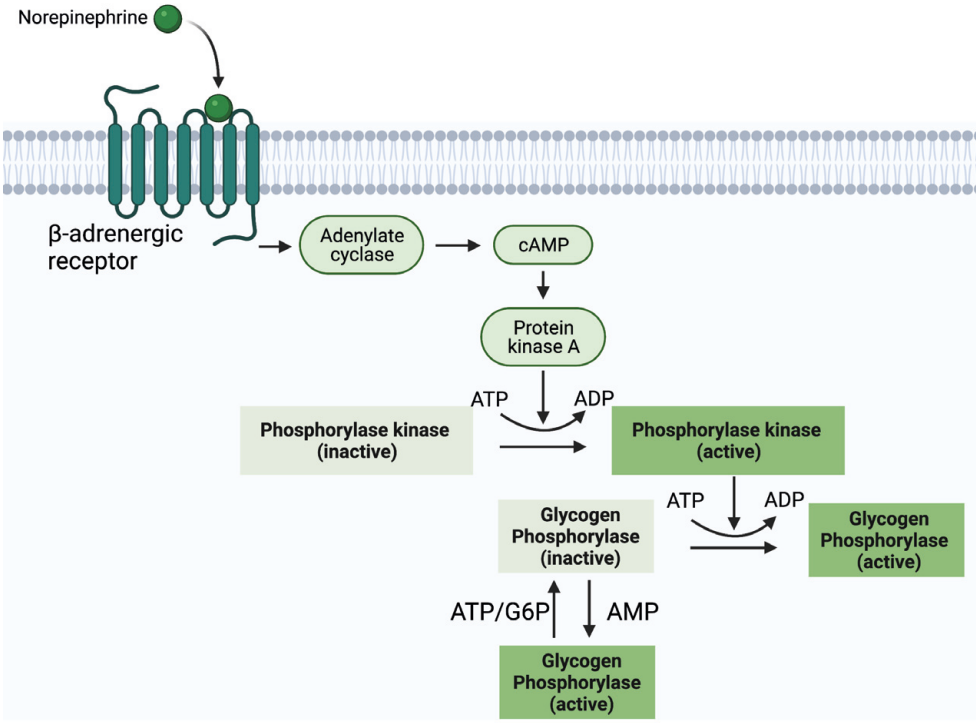

Figure 6. Regulation of Glycogen Phosphorylase. Norepinephrine binds to β-adrenergic receptors, which elevates cyclic AMP (cAMP) and activates Protein Kinase A. Protein Kinase A further phosphorylates Phosphorylase kinase to its active form, which results in the phosphorylation of Glycogen Phosphorylase into its active form. Allosteric regulations of Glycogen Phosphorylase can be initiated by AMP, leading to activation or by ATP or glucose-6-phosphate (G6P), leading to the inactive form of Glycogen phosphorylase. Created with BioRender.com

Dysfunctional glycogen storage

Impairment of glycogen metabolism can contribute to the formation of polyglucosan bodies (PGB) such as Lafora bodies and Corpora Amylacea. The former is seen in neurons of individuals with a severe inheritable form of epilepsy called Lafora disease. The disease is caused by mutations in the Laforin and Malin complex, associated with glycogen synthesis, causing an accumulation of glycogen in neurons. This leads to epilepsy, cognitive impairments and finally death (146). Corpora Amylacea can be found within neurons and astrocytes in the elderly and individuals with neurodegeneration and contains polysaccharides with fewer branches than glycogen (147, 148). This makes them non-degradable by GP, but could in theory be degradable by the enzyme alpha (α)-amylase (149).

Alpha-amylase

Alpha-amylase is known to be the most prominent glycogen-degrading enzyme within the human body. It is primarily produced in salivary glands and pancreas, from where it is secreted into the mouth or in the gastrointestinal tract. In these compartments, it degrades polysaccharides (carbohydrates) into shorter sugar molecules, such as maltose and glucose, by randomly hydrolysing (cleaving) the α1-4 glycosidic bonds within the polysaccharide (Figure 7) (150). However, α-amylase has also been found to be endogenously expressed in twenty different human organs, for example in the liver, kidney, and the thyroid gland (151). The function of α-amylase within these organs is largely unknown, but a study on human and rat livers indicates that it also has glycogen-degrading properties in that organ (152-154). Further, salivary α-amylase appears to have additional functions in the saliva, apart from polysaccharide degradation, including binding to enamel and bacteria (155, 156). The function of these binding properties is not fully understood, but it can lead both to the elimination of bacteria from the mouth as well as a formation of dental plaques (157, 158). Salivary α-amylase has also been shown to be endogenously expressed in rat circumvallate papillae (taste buds), where it might play a role in receptor signaling (159). Additionally, α-amylase expressed in intestinal epithelial cells appears to be important for the proliferation and differentiation of the small intestinal epithelial cells (160).

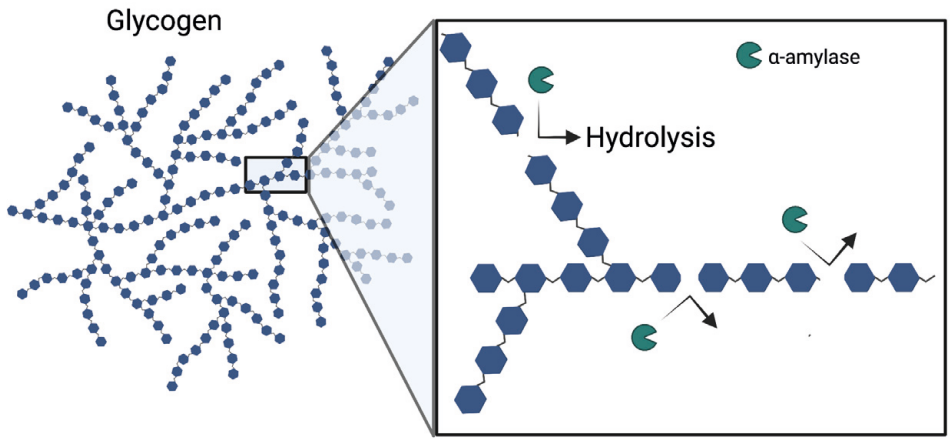

Figure 7. The action of a-amylase on glycogen. α-amylase hydrolyses the α1-4 glycosidic bonds within the glycogen molecule, resulting in the production of maltose and glucose. Created with BioRender.com

The human alpha-amylase genes

The human α-amylases are expressed as several different isoenzymes, encoded by the genes *AMY1A, AMY1B, AMY1C* (salivary α-amylase AMY1A), *AMY2A,* and *AMY2B* (pancreatic α-amylase AMY2A and AMY2B). The sequence homology between these genes is very similar with about 99.9% similarity between the three salivary α-amylase genes, 93.3% between *AMY1A* and *AMY2A*, 93.6% between *AMY1A* and *AMY2B,* and 94.0% between *AMY2A* and *AMY2B*. The α-amylase loci are situated at chromosome 1, and the gene cluster can be highly repetitive (161, 162). Copy numbers (CN) of the human salivary α-amylase *AMY1A* gene can be found in between 2-17 copies among individuals, while the *AMY2A* gene can be found with copy number variants (CNV) of 1 to 4 (161). Studies have found that both *AMY1A* and *AMY2A* CNV correlate with α-amylase activities in plasma (161-165) and that individuals with high *AMY1A* CNV have lower Body Mass Index (BMI), are less prone to develop diabetes, and have a lower postprandial glycaemic response (162, 163, 165-170). In addition, obesity is not seen in individuals with very high *AMY1A* CNV (>10) (167). However, contradictory studies find no correlation between BMI and *AMY1A* CNV (171-174).

Structure and regulations

α-amylase is a monomeric protein, which is folded into a structure with three domains named A, B, and C. The enzyme is dependent on the binding of chloride and calcium for stability and function, but it is the binding of the polysaccharide to the enzymatic cleft that activates α-amylase (175). The catalytic and polysaccharide binding site of α-amylase, as well as its essential chloride-binding site, is located in the A domain. The calcium binding site is found in the B domain whereas the C domain contains glycosylation sites. Further, the N-terminal of α-amylase contains a secretion signal peptide, identified as pyroglutamine (156, 176, 177). The protein sequence homology between salivary and pancreatic α-amylase is 97% where some of the differences are found in the substrate-binding cleft, probably contributing to differences in substrate binding (156, 178).

Rationale

The ability to form memory constitutes the foundation of our self-awareness, social life, and knowledge of our world. Losing this capability, as happens in AD, is devastating, not only for the victim but also for surrounding friends and families. Understanding the underlying mechanisms of memory formation is thus of crucial importance, as the knowledge can direct us towards future therapies to counteract disorders causing memory impairment. However, formation of memory is a very complex event, and we are still only just beginning to understand its basics. But we do know that specialised synaptic signaling in the hippocampus plays a vital role. This signaling requires an enormous amount of energy, which needs to be constantly available. Lately it has become apparent that the storage of glucose, in the form of glycogen, in astrocytes and neurons could function as a back-up system securing energy access. Although the mechanism implicated in brain glycogen degradation has been studied for decades, we have still not uncovered all the actors in this event. The human body meets its energy needs by degrading polysaccharides in food, executed by the efficient and fast-acting enzyme α-amylase. Whether this enzyme could play a role in brain glycogenolysis, and memory formation has yet to be studied.

Aim

Investigate if α-amylase is endogenously produced in the brain, and if so, explore its functions and its roles in Alzheimer's dementia.

Specific aims of the thesis

Paper I: To determine whether α-amylase is expressed and is active in the hippocampus and if the expression is altered in AD patients.

Paper II: Investigate the role of α-amylase in astrocytic glycogenolysis in the presence of amyloid beta.

Paper III. Investigate the relationship between *AMY1A* copy numbers and AD, memory performance and brain α-amylase activity.

Paper IV: Investigate the cellular localization and function of neuronal α-amylase.

Methodology

To conduct the investigations presented in this thesis, a wide range of materials and methods were used. Here I will discuss the relevance, verifications, and limitations of some of them, but for a more detailed description of the methodology, the reader is referred to the Papers included in the thesis.

Human post-mortem tissue samples

Human post-mortem hippocampal tissues (analyzed in **Paper I-IV**) were accessed through a collaboration with the Netherlands Brain Bank (NBB). Written informed consent for using the tissue and clinical data for research purposes was obtained from all patients or their next of kin following the international declaration of Helsinki and Europe´s Code of conduct for Brain Banking. The procedures linked to the brain tissue collection were approved by the medical ethical committee of VU Medical centre Amsterdam and the regional ethical review border in Lund approved the studies. Samples from two different cohorts were analyzed in the studies.

Cohort 1

Hippocampal and EC samples from non-demented controls (NC) (*n=13*) and AD patients (*n=17*). The samples were postfixed according to a protocol previously set up and evaluated in collaboration with NBB in order to obtain optimal immunostaining. The critical step in this protocol is that the samples are fixed in paraformaldehyde (PFA) directly after autopsy for no more than 20h, since over-fixation with PFA affects antigen in the samples. The samples where thereafter section into 40um sections (**Paper I and IV**).

Cohort 2

Hippocampal and EC samples from NC (*n=8*) and AD patients (*n=12*). These samples were used in studies aiming to analyze the relationship between gene expression, protein concentration, activity, and immunostainings of α-amylase and thus homogenates as well as sections were required. The sample was therefore

frozen directly after autopsy and, upon arrival, divided into two 3 mm thick pieces. One piece was incubated in PFA (4%) for 4h and sectioned into 40um sections. The other piece was dissected into CA1, intermediate CA1, and subiculum and homogenized for either mRNA or DNA purification or protein analysis (**Paper I, Paper II, and Paper III**).

Limitations

The major limitation of the studies performed on post-mortem hippocampal tissue (**Paper I-IV**) is the size of the cohorts. A small cohort size can contribute to the risk of over- or under-interpreting the results. Another limitation is the post-mortem delay as biological activity continues after death and may contribute to the degradation of proteins and affect the quality of tissue and thereby the analyzes. Working with post-mortem tissue also comes with the interference of disease comorbidities as all individuals have died of one or another reason. Finally, studies on post-mortem brain tissue only capture a snapshot of events occurring over time and thus do not reveal ongoing processes.

Cell cultures

Four different types of cell cultures were used in the studies presented in this thesis; Human fetal primary astrocytes (**Paper II**), neuroblastoma cell-line SH-SY5Y, neurons derived from human induced pluripotent stem cells (hiPSC) and primary mouse neurons (**Paper IV**).

Before we initiated our experimental studies, we verified that the astrocyte and neuronal cell cultures actually produce α-amylase by performing immunostainings, as well as RT-qPCR and α-amylase activity analyses.

Cells isolated from tissue seldom contains only the desired cell type. This was also the case with the human fetal primary astrocytes bought from ScienCell. We, therefore, purified them using a cell sorting method (MACS) to yield a cell culture containing over 90% astrocytes to make sure that the obtained results herald from astrocytes and not from other cell types. The purity of hiPCS neuronal cultures was determined to contain approximately 90% glutamatergic neurons. The primary mouse neuronal cultures comprised of both neurons and astrocytes, and therefore, the neuronal identity in these cultures was confirmed by immunostainings against neuronal specific markers.

Limitations

The three different neuronal cell cultures have each of them their own strengths but also limitations. The primary mouse neurons are ideally for immunostainings and functional assays since they have fully grown dendritic spines and can communicate

in culture. However, they are not of human origin and given that mouse α-amylase are found only in one isoform it may differ in function. Hence, we may overlook important factors by using these cells. The hiPSC derived neurons have the advantage of being of human origin, however, they express few true synapses, which inevitably limits our investigations on synaptic α-amylase in cells with FAD mutations. The SH-SY5Y cell line is a human neuroblastoma cells-line deriving from a peripheral nerve and is thus less similar to the neurons in the brain. But in contrast to hiPSC and primary mouse neurons, they grow fast and in larger quantities and importantly they can, unlike the other neuronal models, be transfected with siRNA. Finally, since the cell cultures used in these studies are not 100% pure, we cannot exclude the possibility that our results are influenced by the impact of the different stimuli on other cells in the cultures.

Immunostaining

Immunostaining procedures were performed on both human post-mortem hippocampal tissue (**Paper I, II and IV**) and cultured astrocytes (**Paper II**), neuroblastoma SH-SY5Y cells, hiPSC neurons and primary mouse neurons (**Paper IV**). The human post-mortem hippocampal tissue was stained with both immuno-histochemical and immuno-fluorescent staining and the fixated cultured cells were stained with only immunofluorescent protocols. The principle behind immunostaining techniques is to let antibodies with specific epitopes bind to a protein or the antigen of interest in tissue or cells. The antibody-antigen binding can thereafter be visualized by adding a secondary antibody labeled with a detection molecule (fluorescent or peroxidase) that binds to the complex. These methods are highly valuable for detecting and analyzing localizations of specific proteins in tissue or cells. However, the antibodies used in these studies can cause unspecific binding and therefore it is important to verify the results with additional tests. In this thesis, several primary antibodies were used (Table 1).

Table 1. List of primary antibodies in the thesis

Antibody	Specie	Dilution		Distributor	Used in paper
		IHC/IF	WB		
AMY1A	Rabbit	1:75	1:1000	Thermo Fisher. S.	Paper I-IV
AMY2A	Rabbit	1:75	1:1000	Thermo Fisher. S.	Paper I,II
AMY2B	Rabbit	1:75	1:1000	Thermo Fisher. S.	Paper IV
Salivary-α-amylase	Sheap	1:100	1:2000	Abcam	Paper I, IV
GFAP	Mouse	1:100	N	Dako	Paper I
GFAP	Rabbit	1:100	N	Dako	Paper II
NFTL	Mouse	1:50	N	Thermo Fisher. S.	Paper I
P-tau (Thr181)	Rabbit	1:200	N	Santa Cruz	Paper I
Aβ (6E10)	Mouse	1:5000	N	Covance	Paper I, II
Glycogen (ESG1A9)	Mouse IgM	1:200	N	Gift, Prof. Hitoshi Ashida*	Paper II, paper IV
Synaptotagmin	Guinea pig	1:200	N	Synaptic systems	Paper IV
CAM kinase II Clone 6G9	Mouse	1:200	N	EDM Millipore	Paper IV
MAP2	Mouse	1:10 000	N	Sigma Aldrich	Paper IV
GAPDH	Mouse	N	1:10000	Bio Signalling	Paper I, Paper IV

*Kind gift from Professor Hitoshi Ashida at Kobe University, N= Not used

Verification of α-amylase antibodies

Several of our results were based on the use of antibodies directed against α-amylase. Two of them, directed against α-amylase 1A (AMY1A) and 2A (AMY2A), were foremost used and were therefore verified as described below.

If several different antibodies against α-amylase yield a similar staining pattern, it is more likely that the antibodies do in fact detect α-amylase. We therefore stained human hippocampal tissue with antibodies directed against native salivary α-amylase and native pancreatic α-amylase. The native salivary α-amylase yielded a similar pattern as the AMY1A did and the pattern seen after native pancreatic α-amylase staining resembled the AMY2A staining pattern. Also, primary mouse neurons were stained against native salivary α-amylase antibody and an antibody directed against pancreatic AMY2B, and the results showed a similar staining pattern as with AMY1A. Control staining with only secondary antibodies further showed that the yielded staining was not due to unspecific binding of the secondary.

To further verify the AMY1A and AMY2A antibody specificity, Western blot analyzes were done with homogenized hippocampal brain tissue, standard porcine pancreatic α-amylase and human salivary α-amylase (Sigma Aldrich). The membranes were stained with the anti-AMY1A or anti-AMY2A antibodies. Bands of size between 60-100 kDa (α-amylase size is reported to be between 50 and 70 kDa) could be seen in the well loaded with brain samples and a band of size around 60 kDa in the wells loaded with the two α-amylase control samples. In addition, we immunoprecipitated α-amylase from human brain homogenates using AMY1A. The eluted precipitate was thereafter analyzed using Western blot and staining

against both AMY2B antibody or native salivary α-amylase antibody revealed a band at the expected α-amylase size 60 kDa.

Limitations

Despite the verifications and evaluations of the antibodies used in our studies, we cannot entirely exclude the possibility that some of the antibodies capture additional antigens. More advanced analysis is thus required in order to fully trust staining investigating the cellular localization of α-amylase in the brain.

Detection of alpha-amylase

The analysis of α-amylase in human hippocampal tissue and cell cultures was done by the use of several different detection methods. For analyzing α-amylase protein concentration, two types of ELISA were used, one sandwich ELISA from (Nordic Biosite) (**Paper I**) and one in-house developed indirect ELISA (**Paper IV**). The in-house developed ELISA is based on the detection of α-amylase by the AMY1A antibody. The ELISA was carefully evaluated to make sure of reproducibility and avoid unspecific binding. The variation between the data points within the assay (intra-assay coefficient) was calculated to be 12.2 % and the variation between different assay (inter-assay coefficient) was 13.7%. The specificity of the assay was evaluated using two α-amylase standards and the enzyme pectinase as a negative control.

The α-amylase activity within hippocampal tissue (**Paper I, III**) and in cultured astrocytes (**Paper II**) was also analyzed. This was done using a α-amylase activity kit colorimetric (Abcam). Finally, the endogenous gene expression of α-amylase in hippocampal tissue was analyzed using quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). This method measures the gene expression based on how much mRNA the cells have produced. First, mRNA was purified from the sample and by the use of reverse transcriptase it was convert into cDNA, which can be amplified and measured quantitatively in real time with PCR.

The potential pitfalls in RNA purification are the risk of genomic DNA contamination. To verify that the samples are not contaminated, the converted cDNA samples are analyzed together with a control RNA sample not converted with reverse transcriptase. A chain reaction in the control sample indicates contamination with genomic DNA.

Limitations

Due to the close sequence homology between the α-amylase isotypes, some of our detection methods can probably not distinguish between the different isotypes. Our Western blot analysis showing that both the AMY1A and AMY2A antibodies detect salivary and pancreatic α-amylase, exemplify this methodological problem. Hence,

it is not possible to completely be sure of which isoform is measured in, for example, the α-amylase ELISA or the qPCR assay. Furthermore, since the studies in this thesis focused primarily on finding the purpose of α-amylase in hippocampal cell function and AD, we did not investigate the impact of other glycogen degrading enzymes in parallel. We therefore cannot exclude the possibility that the readout i.e., glycogenolysis in response to Aβ and AD pathology, is affected by and impact of other glycogen degrading enzymes such as GP or DE.

Silencing and inhibiting of alpha-amylase

To explore the function of α-amylase, we either inhibited the activity of the α-amylase or silenced the enzyme in our cell cultures. The α-amylase activity was inhibited by either Tendamistat (SH-SY5Y, primary mouse neurons) or Acarbose (astrocytes). Tendamistat is a small molecule (7.9 kDa) that tightly binds to α-amylase and thereby causes a steric hindrance for the substrate (glycogen) to bind to the enzyme (**Paper I**). Acarbose is a competitive substrate for glycogen and by binding to the enzymatic cleft of α-amylase, it inhibits the enzyme (**Paper II**). Silencing α-amylase in SH-SY5Y was done by transfecting small interfering RNAs (siRNA) into the cells. These siRNAs bind to the translated mRNA of interest (α-amylase) and degrade it. To verify that the transfection was successful and the gene sufficiently silenced, RT-qPCR was performed, which showed a 40% reduction of α-amylase gene expression (**Paper IV**).

Limitations

Both α-amylase inhibitors have disadvantages that need to be taken into account. Tendamistat has to our knowledge, never been used in cell-culture studies. Therefore, we cannot by certainty know that the neurons actually take up the peptide. The disadvantage of Acarbose is that it can easily dissociate from the enzyme if the medium is changed, therefore it is difficult to measure the reduction of a-amylase activity in an activity assay. Another disadvantage with Acarbose is that it also inhibits α-glucosidase, an enzyme known to degrade glycogen within lysosomes. We can thus not exclude the possibility that also α-glucosidase is inhibited and may affect the results.

Aβ preparation

Preparations of human synthetic amyloid beta 1-42 (AlexoTech) were made according to Brännström et. al 2014 (179). To be sure that the correct aggregation form was obtained in the preparations, Transmission electron microscopy (TEM), at the Microscopy Facility at the Department of Biology, Lund University was performed.

Limitations

The peptides used in our studies are synthetically manufactured and may thus differ both in aggregation and physiological properties compared to the naturally occurring peptides. In addition, since the cells are stimulated for 18-24 h with the Aβ preparations, it is plausible that oligomers continue their aggregation into fibrils during this time. The preparations can also contain monomers and this potential impurity influences the possibility to distinguish the impact of the different aggregation forms.

Malmö Diet and Cancer Study cohort

The population-based study cohort Malmö Diet and Cancer Study (MDCS) was used for analyzing associations between AMY1A copy numbers, AD, and memory (**Paper III**). MDCS is a prospective study where the baseline examinations were performed between 1991 and 1996. The study was approved by the ethical committee at Lund University (LU 51-90), and all participants provided written informed consent. There were 28,098 individuals participating in the study, and of those, 50 % of the participants invited between 1991 and 1994 were randomly selected for the cardiovascular sub cohort (n=6103) (180). Within the sub cohort, 5422 individuals were genotyped for AMY1A copy number state. At baseline, the participants answered a questionnaire and had a clinical examination (181). Data on AD diagnoses (n=247) until December 31, 2014, were retrieved from the Swedish national patient register and validated in medical records. At re-examinations 2007 to 2012, a sub fraction of 791 individuals completed the Montreal cognitive assessment (MoCA). Data and information about age, sex, APOE e4 status, BMI, education, fasting blood glucose, and diabetes was retrieved from the MDCS cohort. The data collection procedures have previously been described (182, 183).The MDCS cohort analyzes were performed together with Dr. Anna-Märta Gustavsson.

Earlier population-based on α-amylase CN have used different approaches when dividing the cohort before analysis. For example, dividing into three groups containing individuals with high, low and medium CNV (165) or quartiles with a similar number of individuals in each group (184). Since we were interested in the impact of high *AMY1A* CNV on AD risk and memory performance (in regard to studies demonstrating a beneficial impact on BMI in individuals with *AMY1A* CNV over 10), we divided the individuals into four groups; low (1-5), reference (6), high (7-9) and very high (≥10) CN. The group with 6 *AMY1A* CN was chosen to be the reference group since this CNV is the most common. The association between *AMY1A* CNV and the development of AD was analyzed with Cox regression models, which in this case analyzed the risk (Hazard ratio (HR)) of being diagnosed with AD during a specific time (20 years). Death and another dementia diagnosis were treated as a competing risk, meaning that the HR of AD was estimated in

individuals who were alive and not diagnosed with another dementia. (**Paper III**). Since age, sex, education, BMI, fasting blood glucose, diabetes and *APOE* ε4 are risk factors for AD, it is important that these variables are used as covariates in the statistical analysis in order to reduce their influence on the result.

Limitations

The recommended size of a population-based study is 10% of the population (in this case, 10% of the inhabitants of Malmö), which the MDCS cohort constitutes. The cohort size of the 5422 individuals included in our study should therefore be considered small and it consequently contains few cases of AD. This may introduce statistical biases and false results.

Studies performed by collaborators

- The culturing and differentiation of hiPSC cells were performed by Henriette Haukedal at Associated professor Kristine Freudes laboratory, The University of Copenhagen.
- The isolation of primary mouse neurons and calcium imaging analysis were performed by Dr Isak Martinsson at Professor Gunnar Gouras laboratory, Lund University.
- The Genotyping of *AMY1A* CNV (**Paper III**) was performed by TATAA Biocenter (Gothenburg, Sweden)

Main Findings

Alpha-amylase is present in human brain

The initial aim of this thesis was to investigate the presence of α-amylase in the human brain. By the use of four different α-amylase detection methods; immunostainings, ELISA, gene expression analysis, and α-amylase activity assay, we showed that α-amylase is expressed, produced and active in the human brain (**Paper I**). Interestingly, the activity of α-amylase appeared to be gender-dependant as we found higher α-amylase activity in females compared to males (**Paper III**).

Alpha-amylase is found in neurons

To further identify the cell types producing α-amylase, we immunostained hippocampi from non-demented individuals against α-amylase isotype AMY1A. The staining yielded an interesting pattern, which resembled structures corresponding to dendritic spines (Figure 8A) (**Paper I and Paper IV**). Double immunostaining against cell neuronal-specific markers and α-amylase could confirm that the structures were indeed neurons (Figure 8B) (**Paper I**).

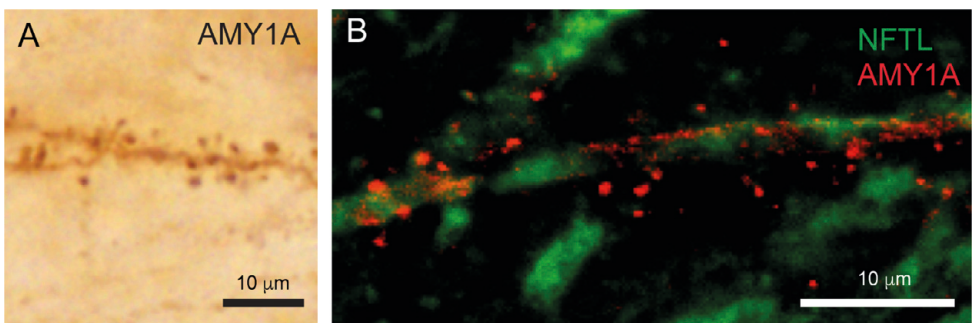

Figure 8. Localization of alpha (α)-amylase in human hippocampal neurons. The image in (A) shows α-amylase immunoreactivity in structures resembling dendritic spines in Cornu Ammonis (CA1) of a non-demented control. Image in (B) shows that the α-amylase positive structures are associated with the neuronal marker neurofilament light chain (NFTL).

Detection of α-amylase in neuronal dendrites inspired us to investigate its localization and expression in neurons in more detail. For this purpose, we used three neuronal cell culture models; SH-SY5Y, hiPSC-neurons, and primary mouse neurons. All three neuron types showed presence of α-amylase, verified either by immunoreactivity, gene-expression, or protein concentration. Additionally, staining of primary mouse neurons confirmed that neuronal α-amylase can be found in dendrites and is closely associated with synaptic boutons (Figure 9B-D) (**Paper IV).**

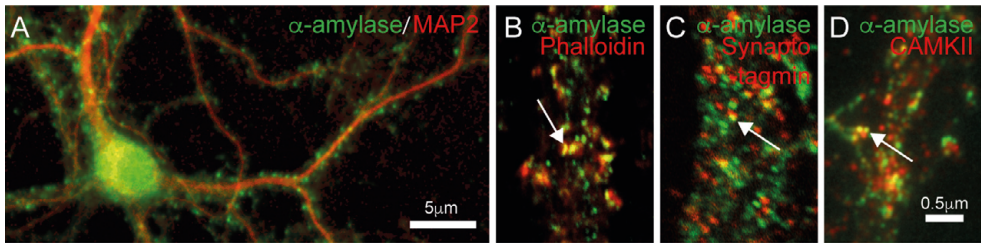

Figure 9. Localization of alpha (α)-amylase in primary mouse neurons. The image in (A) shows that α-amylase (green) is associated with neuronal marker MAP2 (red). Image in (B-D) demonstrates an association between α-amylase (green), the F-actin marker Phalloidin (red) (B), the synaptic markers Synaptotagmin (red)(C), and CAM kinase II (CAMKII) (red) (D) (indicated with arrows).

Alpha-amylase is found in astrocytes

We also immunostained the hippocampal tissue with an antibody directed against AMY2A. Interestingly, this staining yielded a different pattern compared to AMY1A, and instead, cells with a glial morphology appeared positive. These α-amylase positive glial cells were confirmed to be astrocytes (**Paper I**). Interestingly, α-amylase was foremost found in astrocytes with hypertrophic cell bodies, indicating that the enzyme is upregulated when astrocytes are activated (Figure 10A-C). The astrocytic capability to produce α-amylase was further confirmed in cell culture studies, where both expression and activity of the enzyme in cultured astrocytes could be detected (**Paper II**).

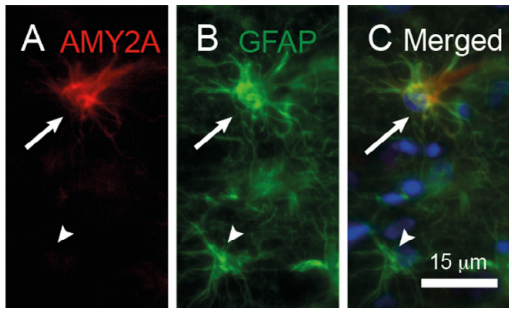

Figure 10. Alpha (α)-amylase is expressed in activated astrocytes. Immunofluorescent staining of post-mortem hippocampal tissue using the α-amylase antibody AMY2A (red in A) and astrocyte antibody GFAP (green in B) shows co-expression (A and B is merged in C) in astrocytes with a hypertrophic morphology (arrow). The AMY2A immunoreactivity is not detected in astrocytes with a resting morphology (arrowhead).

Alpha-amylase is associated with glycogen degradation

The finding of astrocytic and neuronal α-amylase triggered the question of its function within these cells. Since the main function of α-amylase in the periphery is to degrade glycogen, and given that we found α-amylase in cells previously reported to produce glycogen (neuron (130, 185) and astrocytes (132, 136)), we hypothesized that the enzyme has a similar role in the brain. We were able to detect glycogen in astrocytes in post-mortem hippocampi as well as in cultured astrocytes, SH-SY5Y cells, hiPSC-neurons, and primary mouse neurons (Figure 11A-C) (**Paper I, II and IV**). The glycogen content in SH-SY5Y increased when α-amylase expression and activity was silenced, which supports the idea that neuronal α-amylase degrades neuronal glycogen (**Paper IV**). Also, when α-amylase was inhibited in cultured astrocytes, a major decrease in released lactate was seen, indicating that a large part of the glucose used for glycolysis depends on α-amylase degradation of glycogen in astrocytes (**Paper II**).

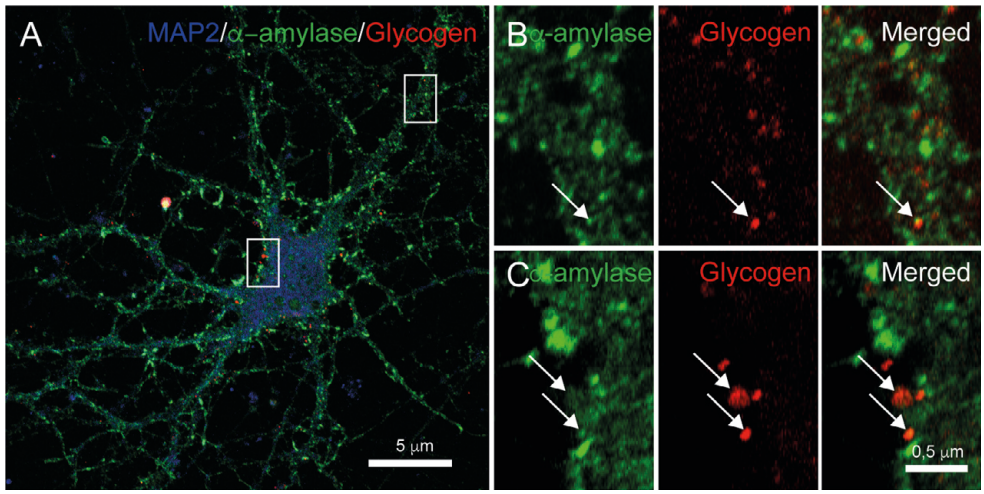

Figure 11. Glycogen in primary mouse neurons. Confocal image in (A) shows Immunofluorescent staining of alpha (α)-amylase (green) and glycogen (red) in primary mouse neurons. The white squares indicate the magnified areas seen in (B) and (C), where close association between α-amylase and glycogen can be seen (arrows).

Alpha-amylase is involved in Neuronal signaling

The discovery of neuronal α-amylase in dendrites, and synapses, indicates a role for α-amylase in neuronal signaling. We therefore analyzed calcium oscillation in primary mouse neurons after inhibiting α-amylase activity. Interestingly, the results showed that inhibition led to lower amplitude of intracellular calcium concentrations and lower inter-spike intervals (Figure 12A-D). This finding suggests altered neuronal signaling and a dysregulated calcium homeostasis when α-amylase activity was reduced (**Paper IV**).

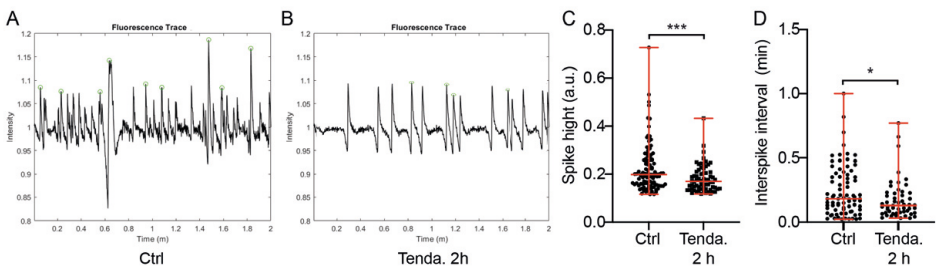

Figure 12. Calcium imaging of Tendamistat treated primary mouse neurons. The traces in (A and B) show the spiking activity of primary mouse neurons labeled with Fluo4 AM (the calcium indicator) after stimulation with vehicle (Ctrl) (A) or Tendamistate (Tenda) for 2 h (B). The graph in (C) demonstrates significantly lower amplitude of calcium concentrations (spike height) after 2 h stimulation with Tendamistat compared to Ctrl. The graph in (D) demonstrates significantly shorter inter-spike intervals after 2 h Tendamistat stimulation compared to Ctrl. Statistical analysis was performed by Mann–Whitney U test, and data is presented as median and range ***indicates p-value < 0.001 and *indicates *p*-value < 0.05

Alzheimer's dementia alters the presence of alpha-amylase

The next aim of this thesis was to investigate the role of α-amylase in relation to Alzheimer's disease. This was done by analyzing hippocampal post-mortem tissue from both AD patients and non-demented controls, cell cultures of astrocytes and neurons, as well as analysis of genetical variations of *AMY1A* in a population-based study cohort (**Paper I-IV**). By comparing α-amylase gene expression between AD and non-demented controls (NC), we found that α-amylase gene expression is decreased in AD patients. This decrease was additionally associated with an increase in Aβ and NFT load. Surprisingly, α-amylase protein concentration and activity were instead found to be upregulated in AD patients compared to NC (**Paper I**).

Neuronal alpha-amylase is decreased in AD

To investigate how AD pathology specifically affects neuronal α-amylase, we analyzed the presence of α-amylase in dendritic spines of AD patients. We found that dendritic α-amylase in hippocampus was observed to a lesser extent in AD brains compared to NC (Figure 13A-D) (**Paper I**). This observation was confirmed to be statistically significant in studies in a larger cohort, and the α-amylase decrease correlated with neuropathological evaluations of Aβ and NFT (Figure 13E-H) **(Paper IV**).

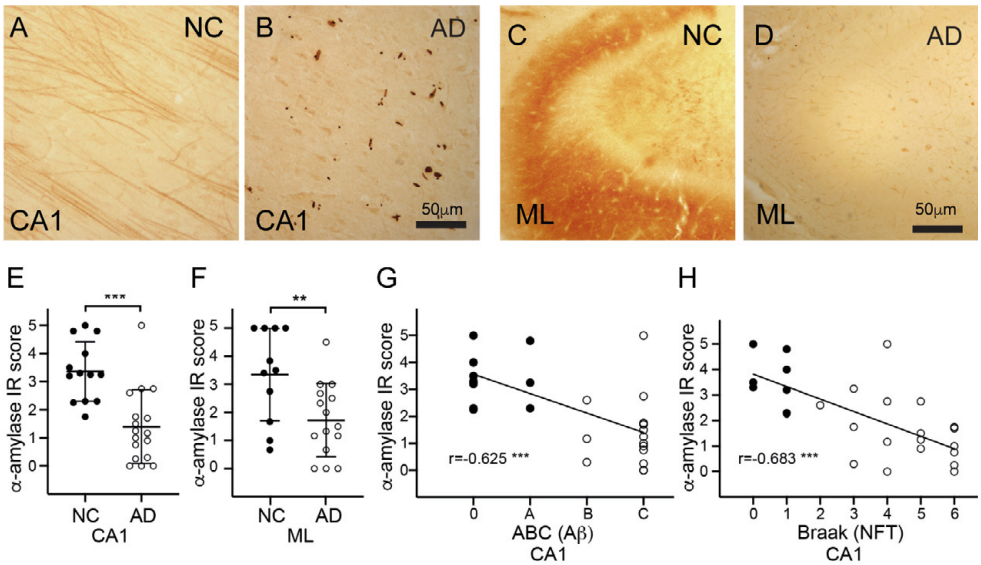

Figure 13. Alpha (α)-amylase in hippocampal neurons is reduced in patients with Alzheimer´s Disease. Immunohistological stainings against α-amylase AMY1A (A-D) show reduced immunoreactivity in the Cornu Ammonis (CA1) and molecular layer (ML) of Alzheimer's dementia patients (B and D, respectively) compared to non-demented controls (NC) (A and C, respectively) Graph in (E and F) shows scores of α-amylase immunoreactivity (IR) in dendritic spines (DS) in CA1 (E) and scores of grained α-amylase IR in the molecular layer (ML) (F), which both are significantly higher in NC compared to AD. Scatter plots in (G and H) demonstrate a negative correlation between DS α-amylase IR in CA1 and amyloid beta (Aβ) ABC scores (G) and neurofibrillary tangles (NFT) Braak scores in (H). Graphs in (E and F) are presented as mean±SD, and statistical analysis was done using *t* test. The correlation analyzes in (G and H) were done using Spearman correlation test. ***indicates *p*-value<0.001, **indicates *p*-value<0.01

To investigate if loss of neuronal α-amylase in AD patients is due to a direct impact of Aβ, we exposed SH-SY5Y cells to oligomeric Aβ1-42. We found that the Aβ exposed neurons had significantly lower α-amylase concentrations. Also, hiPSC-neuron with PSEN1 mutation (causing higher Aβ production) showed reduced α-amylase compared to its isogenic control (**Paper IV**). This may imply that the reduction of neuronal α-amylase in AD hippocampus is a direct effect of the AD characteristic accumulation of Aβ. Surprisingly, both Aβ-exposed SH-SY5Y cells and hiPSC-neuron with PSEN1 mutation showed lower levels of glycogen granules, which is inconsistent with the previous finding demonstrating an increased glycogen load in neurons with silenced α-amylase. However, Aβ is known to influence pathways implicated in glycogen formation (186, 187). Therefore, it is likely that Aβ affects glycogen and α-amylase by two parallel but different pathways.

Astrocytic alpha-amylase is increased in AD

Since we observed α-amylase in reactive astrocytes, we continued to investigate whether this observation is linked to AD pathology. Indeed, we found an increased number of α-amylase positive astrocytes in AD patients. These were often located

in close vicinity to Aβ plaques (Figure 14A-C). Additionally, the numbers α-amylase positive astrocytes increased with Aβ and NFT load (**Paper II**), indicating a link between Aβ pathology and upregulation of α-amylase in astrocytes. To further explore this idea, we analyzed α-amylase in astrocytes after oligomeric or fibrillar Aβ1-42 exposure. Analysis showed that foremost Aβ1-42 fibrils led to higher α-amylase activity in the astrocytes (Figure 15A-B). Interestingly, just like in neurons, glycogen levels changed alongside α-amylase, where both, in this case, were increased after Aβ1-42 exposure. Similar findings were noted in hippocampal AD tissue where astrocytes with a high expression of α-amylase contained higher glycogen load compared to astrocytes in NC (**Paper II**). AD patients also showed elevated (trend towards significance) amounts of polyglucosan bodies, which correlated with increased α-amylase activity (**Paper I**). To investigate if the increased α-amylase activity and glycogen levels were linked to glycogen degradation into glucose used in glycolysis, the enzyme activity of pyruvate kinase (PKM) and released lactate levels were measured. The result showed increased PKM but no change in released lactate in the presence of Aβ1-42 (Figure 15C-D). This indicates that increased α-amylase activity leads to an upregulation of the glycolysis but lactate, which according to the ANLS hypothesis, is released to the extracellular space, is not the final product (**Paper II**).

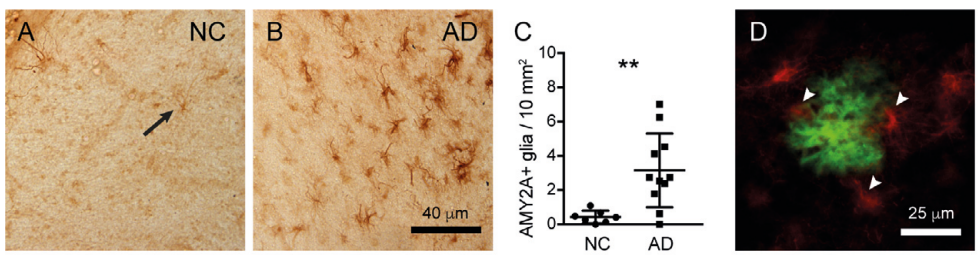

Figure 14. Increased alpha (α)-amylase immunoreactivity in Alzheimer's dementia (AD) patients. Image in (A and B) shows an α-amylase AMY2A immunostaining if entorhinal cortex (EC) in a non-demented (NC) (A) and a patient with AD. Arrow in (A) indicates an AMY2A positive glial cell in the NC, while the staining in (B) revealed a high number of AMY2A positive glial cells in AD patients. The graph in (C) shows the significant higher number of glial cells positive for AMY2A (AMY2A+glia) in AD (n=12) compared to NC (n=8). Data was analyzed using student t-test and presented as mean±SD. ***p*-value >0.01. Image in (D) shows an amyloid-beta plaque (green) surrounded by AMY2A positive glial cells.

Interestingly both increased synthesis of glycogen and upregulation of α-amylase production can be regulated by the binding of norepinephrine to β-adrenergic receptors via signal transduction pathways including cAMP and Ca^{2+}. These receptors are also targets for Aβ binding (188). To investigate if the mechanistic pathway, resulting in the upregulation of α-amylase and glycogen, acts via the β-adrenergic receptors, we stimulated the astrocytes with either oligomeric or fibrillar Aβ1-42 together with the β-adrenergic receptor antagonist Propranolol. The results

showed that indeed the effect of Aβ was attenuated with the presence of Propranolol (Figure 15E-F) (**Paper II**).

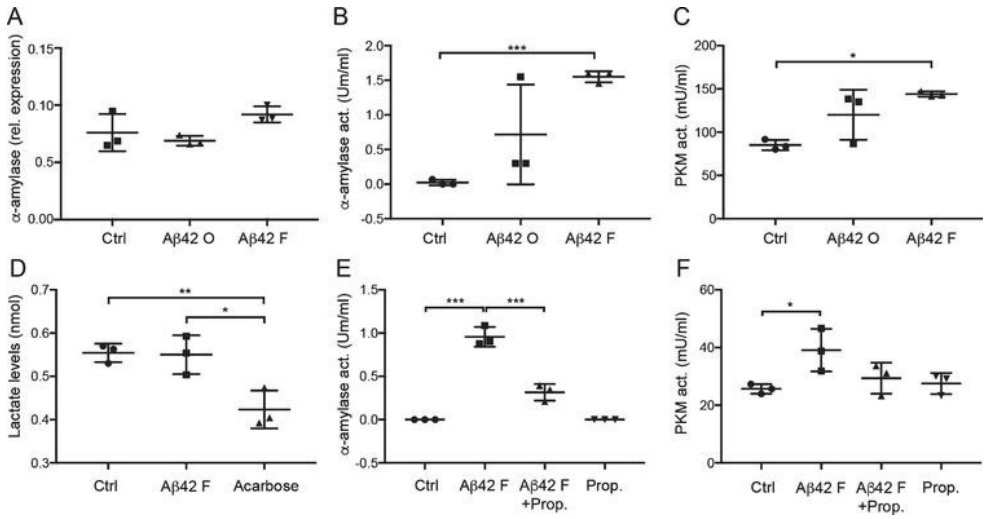

Figure 15. Unchanged gene expression but increased activity of α-amylase and glycolytic changes in cultured astrocytes after Aβ1-42 stimulation. Column scatter plot in (A) shows relative expression of α-amylase normalized against values of housekeeping genes in astrocytes stimulated with control (Ctrl), 10 μM Aβ1-42 oligomers (Aβ42 O), and 10 μM Aβ1-42 fibrils (Aβ42 F). No significant difference was seen between the stimulations. Column scatter plot in (B) shows the change in α-amylase activity seen in stimulated astrocytes. Aβ42 F stimulated astrocytes showed significantly higher α-amylase activity compared with Ctrl. Column scatter plot in (C) shows the change in pyruvate kinase (PKM) activity seen in stimulated astrocytes cells. Aβ42 F significantly increased PKM activity in astrocytes stimulated with Aβ42 F compared with Ctrl. Column scatter plot in (D) shows the unaltered lactate levels in cell culture medium after stimulation with Aβ42 F, but reduced lactate levels after stimulation with the negative control 5 μM acarbose. Column scatter plot in (E) shows the inhibiting impact of 1 μM propranolol (Prop.) on Aβ42 F induced-amylase activity, were propranolol counteracted the Aβ42 F-induced-amylase activity. Column scatter plot in (F) shows the PKM activity in astrocytes after stimulation with propranolol and Aβ42 F. The experiments were performed independently three times with two replicates. Data was analyzed using one-way ANOVA with Tukey post-test and presented as mean ± SD. * *p*-value < 0.05, * * *p*-value < 0.01, * * * *p*-value < 0.001.

High AMY1A copy numbers are associated with lower risk for AD

Although the studies in **Paper I, II and IV** indicate that Aβ pathology alters brain α-amylase expression, we cannot exclude the possibility that it might be the other way around, i.e., that the reduced α-amylase in AD patients, due to genetic predisposition, contributes to the pathology. We therefore investigated the associations between *AMY1A* CNV and the risk for developing AD. We found that individuals with high *AMY1A* copy numbers (≥10) had a lower hazard ratio (risk) for developing AD compared to the reference group (=6) (Table 2). This is in line with other studies, showing that high copy numbers are beneficial for BMI, insulin, and glucose levels (167, 168).

Table 2. Cox proportional hazards of Alzheimer's dementia by number of copies of AMY1A gene

	Alzheimer's dementia HR (95% CI)		
***AMY1* copy number**	Unadjusted	Age-adjusted	Fully adjusted[1]
Per 1 increase	0.98 (0.93, 1.03)	0.98 (0.93, 1.03)	0.97 (0.92, 1.02)
By four groups			
1-5	0.74 (0.53, 1.02)	0.75 (0.54, 1.04)	0.75 (0.54, 1.05)
6 (reference)	1	1	1
7-9	0.93 (0.67, 1.27)	0.92 (0.67, 1.27)	0.86 (0.62, 1.20)
≥10	0.62 (0.41, 0.94)*	0.62 (0.41, 0.95)*	0.59 (0.38, 0.90)*
***n* events/total**	247/5422	247/5422	235/5028

Data is presented as HR (95% CI) [1]adjusted for age, sex, education, *APOE* ε4, body mass index and diabetes at baseline. *n* represents number of events (cases with AD) and total number of individuals included in the model. **p* value<0.05

Since high *AMY1A* copy numbers seem to lower the risk for AD, we further wanted to investigate the relation between *AMY1A* CNV and memory on a sub-group within the MDCS cohort. The results showed that individuals with very high copy numbers (≥10) performed significantly better on episodic memory test (word recall) compared to the reference group (Table 3). Studies on a smaller cohort further showed a relationship (trend towards significance) between α-amylase activity levels and *AMY1A CNV*, indicating that brain α-amylase activity, at least in part, is dependent on *AMY1A* copy numbers.

Table 3. Associations between Montreal Cognitive Assessment test scores and *AMY1A* copy number variation

	MoCA test participants (n=790) Mean ± SD	
***AMY1* copy number**	**Total MoCA score**	**Word recall score**
By four groups		
1-5	25.5 (3.0)	3.0 (1.3)
6 (reference)[1]	25.1 (3.3)	2.8 (1.4)
7-9	25.5 (3.1)[a]	3.1 (1.4)
≥10	25.7 (3.1)	3.2 (1.2)*

Data is presented as mean (SD). Data is analyzed using one-way ANOVA with Tukey post hoc test **p*-value >0.05. [1]The most common AMY1A copy number variant is 6, therefore used as the reference group. [a]Missing data (*n*=1)

Discussion

The results presented in this thesis demonstrate the expression of the enzyme α-amylase in the human brain and specific cell types, which is changed in the presence of AD or amyloid beta. Here I will discuss the implication of these findings in a larger context.

The importance of alpha-amylase in human brain

The physiological role of alpha-amylase

The finding of α-amylase in the human brain raises the question about the enzyme's physiological importance within this organ. Since α-amylase is well-known for its polysaccharide degrading properties, not only in the mammal gastrointestinal tract but also in several other organisms like; plants, fungi, yeast, and bacteria *(189)*, it is easy to assume that a α-amylase would have a similar function in human tissue. Findings demonstrating endogenously expressed α-amylase in the liver, which has a high affinity for glycogen and degrades it directly into glucose (154), support the idea of α-amylase as a glycogen degrading enzyme within tissue. However, one could argue that endogenously expressed α-amylase in human tissues have no physiological functions since its concentrations are so low (151). To compare, α-amylase in the gastrointestinal tract is expressed 100-1000 times higher than in for example the liver (151, 164). But there is a big difference in the amount of substrate in what we eat (dietary carbohydrates) compared to the glycogen found in the different organs. The brain contains only 0.1% glycogen and does therefore not require a high amount of glycogen degrading enzymes. Our studies showed a concentration of α-amylase within the same concentration range as other brain glycogen degrading enzymes (**Paper I)**, such as GP *(190)*. Therefore, it is likely, that even in low concentrations, endogenously α-amylase has a physiological function of degrading glycogen also in the brain, as our studies indicate (**Paper II and Paper IV)**.

Brain alpha-amylase isotypes

Interestingly, the antibodies against AMY1A and AMY2A gave rise to two different staining patterns in hippocampal tissue, indicating a cell-specific of the α-amylase isotypes in astrocytes and neurons. Of note, our Western blot analyzes showed that both antibodies detect salivary and pancreatic α-amylase. It may thus be that the antibodies are more specific towards the tertiary structure of the two isotypes and cannot distinguish between them in a condition where the tertiary structure is altered (SDS-PAGE). The very close sequence homology between the α-amylase isotypes supports this idea. However, it may also be that the α-amylase found in brain is a specific variant, separated from the ones found in the gastrointestinal tract. The brain α-amylase is found intracellularly, in contrast to the secreted gastrointestinal, indicating that it may lack the N-terminal secretion signal molecule pyroglutamine.

The alpha-amylase-glucose pathway

According to the ANSL hypothesis, glutamatergic signaling stimulates astrocytes to degrade glycogen in order to provide neurons with energy. Since G6P (the end-product of GP degradation) cannot be transported over the glial cell membrane, astrocytes convert G6P into lactate. This product can be released by astrocytes and taken up by neurons (118). However, the procedure involves additional costs for the cells as the enzyme lactate dehydrogenase (LDH) needs to convert pyruvate into lactate in the astrocytes, which in the neuron is, by the same enzyme, oxidized (use of $NADH^{+}$) into pyruvate again (123, 124). From this perspective, transportation of glucose would be more efficient for both astrocytes and neurons. But the consensus within the field has been that astrocytes lack the enzyme glucose 6 phosphatase and therefore are unable to produce glucose. This belief has, however, been challenged since G6Pase expression has been found in rat astrocytes (191). Interestingly, α-amylase has the capacity to cleave glycogen directly into glucose (154) and could thus, in theory, produce glucose for transportation out of the cell. This may explain why we did not detect a change in lactate release despite the found upregulation of α-amylase and PKM in astrocytes after Aβ exposure (**Paper II**). The levels of released glucose were not measured in our experiments so whether this idea holds true or not remains to be investigated. But a previous study has shown that astrocytes contain pools of glucose (192). Moreover, it has been shown that stimulation of the β-adrenergic receptors in the rat brain elevates glucose and not lactate levels, while a tail pinch elevates both (193). Given these results and our own findings, it is tempting to speculate that astrocytes provide energy by two pathways, the "GP-lactate pathway" and the "α-amylase-glucose pathway". The former is used in ANLS, but both can be induced when norepinephrine, secreted by projections from locus coeruleus, binds to β-adrenergic receptors on astrocytes. The "α-amylase-glucose pathway" upregulates astrocytic α-amylase, which degrades glycogen into

glucose. Glucose is then transported into the extracellular space, where it can be taken up by nearby neurons (Figure 16).

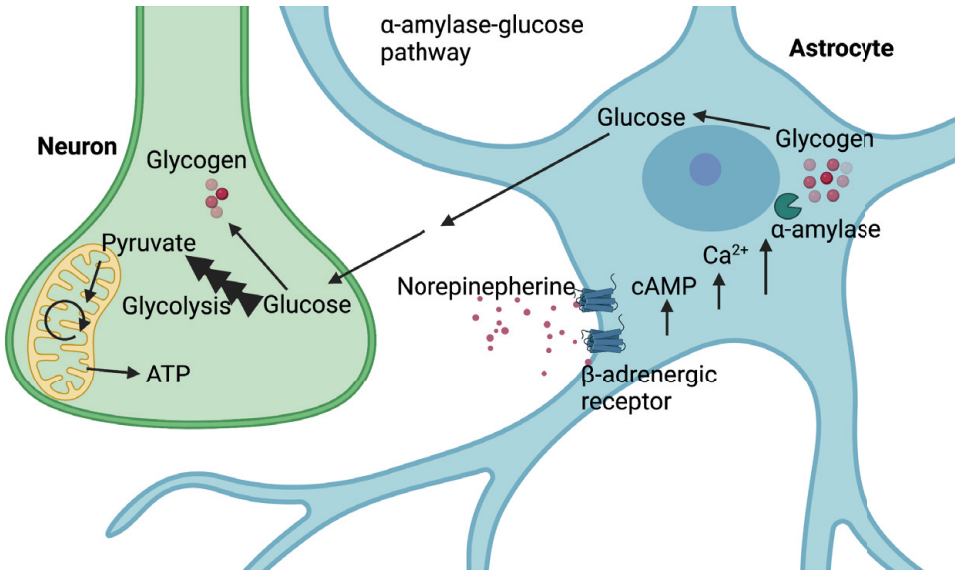

Figure 16. Illustration of the "α-amylase-glucose-pathway" hypothesis. Norepinephrine activates β-adrenergic receptors on astrocytes, which induces a signaling cascade that initiates cyclic AMP(cAMP) and calcium (Ca2+) to elevate. This elevation further leads to the activation of α-amylase and long-term upregulation of glycogen, which induces cleavage of glycogen by α-amylase and elevated glucose level as a result. The increased glucose levels in the astrocytes are released to the extracellular space and taken up by neurons. In the neuron, the glucose can then enter the glycolysis and further TCA-cycle to produce ATP or be synthetized into glycogen. This process might fuel synaptic signaling and the formation of long-term potentiation (LTP). Created with BioRender.com

Alpha-amylase and memory

Our population-based study indicates that individuals with high *AMY1A* copy numbers have better episodic memory, potentially enhancing cognitive resilience and delaying AD onset (**Paper III**). In what way high production of α-amylase might affect memory formation can only be speculated on, but in light of the importance of astrocytic and neuronal glycogen degradation in memory forming LTP (115, 116, 194, 195), it may well be that α-amylase glycogen degrading properties play a role.

The fact that we found an association of α-amylase with synapse markers as synaptotagmin and CaMIIK (**Paper IV**) is particularly interesting as post-synaptic CaMKII is an enzyme specifically involved in the formation of LTP. When calcium influx (amplitude) is elevated upon neuronal signaling, CaMKII becomes activated, resulting in an upregulation of AMPA receptors and strengthening of the synapse (196). But if the calcium influx amplitude is moderate, the AMPA receptors are removed and the synapse is weakened, a term called long-term depression (LTD)

(197-199). In view of this, it is intriguing that our results showed a lower calcium amplitude in neurons when α-amylase activity was inhibited (**Paper IV**). Although further studies are required to confirm that a reduction of α-amylase leads to LTD, it is tempting to speculate that the loss of neuronal α-amylase in AD patients (**Paper I**), induces LTD and thereby play a role in the memory impairments seen in the disease.

Alpha-amylase and AD pathology

Several animal studies have shown that Aβ has a direct negative impact on memory formation (200, 201). Studies on chickens further indicate that the peptide impairs memory by specifically disrupting astrocytic glycolysis by binding to β-adrenergic receptors (186, 202). Our studies show that Aβ acts via β-adrenergic receptors on astrocytes to upregulate α-amylase and glycolysis (**Paper II**), which contradicts the former study. Interestingly, Aβ stimulation also increased glycogen load in astrocytes (**Paper II**), which may counter the idea that α-amylase´s primary function in astrocytes is to degrade glycogen. However, a study has shown that stimulation of β-adrenergic receptors induces both short-term glycogenolysis and long-term glycogenosis in astrocytes (203), and it is plausible that Aβ has a similar effect. The significance of an upregulation of α-amylase in response to Aβ remains to be investigated, but if the "α-amylase-glucose pathway" hypothesis is applied, it is tempting to speculate that astrocytes react upon Aβ by upregulating α-amylase in order to liberate glucose in an attempt to rescue nearby neurons. But Aβ also binds other receptors, such as toll-like receptors, which provokes activation of the astrocytes. Activated astrocytes are thought to be beneficial as they phagocytize Aβ and dystrophic neurites. However, the activation also leads to an upregulation of cytokines and chemokines, which not only recruit additional astrocytes and microglia but also damage neurons. In AD, the astrocytes become dysfunctional where a vicious circle of neuroinflammatory events and glutamate toxicity causes synaptic loss and neuronal death. From this perspective, one may question whether the upregulation of astrocytic α-amylase is beneficial or detrimental in AD. This possible dual role for α-amylase makes it a difficult target for therapy, and more research on whether the upregulation of α-amylase fuels astrocytic activation or is a parallel rescuing event is needed.

Conclusion

The general perception of α-amylase is that it serves as a digestive enzyme for carbohydrates in our gastrointestinal tract. The studies in this thesis expand our knowledge by providing evidence that α-amylase is also present in the brain as well as proposing a role for the enzyme in essential mechanisms implicated in brain glycogenolysis. These findings are novel and important for our future understanding of brain metabolism, memory formation, and AD pathology.

Specific conclusions

- α-amylase is endogenously expressed in the human hippocampus and can specifically be found in the glycogen-producing astrocytes and neurons
- The function of astrocytic α-amylase appears to be particularly linked to glycogen-degradation involved in reactivity of the cell
- Neuronal α-amylase seems to be associated with glycogenolysis implicated in synaptic signaling
- Patients with AD display pathology-dependent alterations in hippocampal α-amylase, with an increased number of α-amylase expressing astrocytes encircling Aβ-plaques and a substantial loss of dendritic/synaptic α-amylase
- The AD related alterations in α-amylase appear to be directly linked to Aβ, as the peptide upregulates α-amylase in astrocytes and downregulates the same in neurons.
- The Aβ induced upregulation of α-amylase in astrocytes is mediated via β-adrenergic receptors, which leads to the degradation of glycogen and theoretically a release of glucose into the extracellular space.
- Genetic predisposition of high α-amylase production may improve episodic memory and reduce the risk of AD.

Future perspectives

The studies in this thesis are the first, to my knowledge, to demonstrate a presence and function of α-amylase in the human hippocampus. These novel findings challenge our current understanding of brain glycogen metabolism, a research field still in its cradle. It is therefore important that studies on α-amylase's role in brain will be perused. Here I will list future research areas that I find important.

- Investigate if α-amylase is expressed in brain specific isotypes, if the identity of such isotypes differs between neurons and astrocytes and whether there are any functional differences between the isotypes.
- Further explore the "amylase-glucose-pathway" hypothesis, by investigating whether a binding of norepinephrine to β-adrenergic receptors elevates α-amylase activity in astrocytes and if this leads to an increased secretion of glucose available for neurons.
- Investigate whether β-adrenergic receptors on neurons affect α-amylase expression and thereby neuronal signaling.
- Investigate if the upregulation of α-amylase in astrocytes is linked to reactivity in general or if it is an Aβ specific event. Also, if α-amylase upregulation precedes and fuels an astrocytic activation or if it is a consequence of the same.
- Investigate if α-amylase might have other functions besides glycogen degradation, for example, binding and storing calcium similar to calbindin.
- Define potential hippocampal alterations in mice lacking the α-amylase genes and investigate if a lack of the gene induces behavioral changes, such as memory impairments.
- Investigate α-amylase as a potential therapeutic target in diseases that accumulates glycogen, for example, Lafora disease or glycogen storage diseases.

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