Für meine geliebten Eltern

Rosi und Dieter Bartl

Impairment of insulin signaling pathway in Alzheimer's disease

Beeinträchtigung des Insulinsignalweges bei Alzheimer Demenz

Doctoral thesis for a science doctoral degree

at Julius-Maximilians-Universität Würzburg

Section: Neurobiology

Submitted by

Jasmin Bartl

from

Böblingen

Würzburg 2012

Submitted on:

…………………………………………………………………….
Office stamp

Members of the graduation committee:

Chairperson: Prof. Dr. Thomas Dandekar (University of Würzburg)

Primary Supervisor: PD Dr. Edna Grünblatt (University of Zürich)

Supervisor (Second): Prof. Dr. Erich Buchner (University of Würzburg)

Date of Public Defence: ………………………………..

Date of receipt of Certificates: …………………………

AFFIDAVIT

(EIDESSTATTLICHE ERKLÄRUNG)

According §4 Abs. 3 Ziff. 3, 5 and 8
of the "Promotionsordnung der Julius-Maximilians-Universität Würzburg"

I hereby declare that my thesis entitled "**Impairment of insulin signaling pathway in Alzheimer's disease**" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Zürich, ………………………………………………………….

“Dissertation Based on Several Published Manuscripts“
Statement on individual author contributions

Publication: Bartl, J., C. J. Scholz, M. Hinterberger, S. Jungwirth, I. Wichart, M. K. Rainer, S. Kneitz, W. Danielczyk, K. H. Tragl, P. Fischer, P. Riederer, and E. Grünblatt (2011) Disorder-specific effects of polymorphisms at opposing ends of the Insulin Degrading Enzyme gene. *BMC Med Genet* 12:151.(DOI 1471-2350-12-151 /10.1186/1471-2350-12-151)						
Participated in	**Author-Initials,** Responsibility decreasing from left to right					
Study Design	ED	PR				
Data Collection	MH	SJ	IW	MKR	WD	KHT
Data-Analysis and Interpretation	CJS	**JB**	SK	PF		
Manuscripte writing	**JB**	CJS				

I confirm that I have obtained permission from the co-authors see signature below:

C.J. Scholz	
M. Hinterberger	
S. Jungwirth	
I. Wichart	
M.K. Rainer	
S. Kneitz	
W. Danielczyk	
K.H. Tragl	
P. Fischer	
P. Riederer	
E. Grünblatt	

Publication: Bartl J, Monoranu CM, Wagner AK, Kolter J, Riederer P, Grünblatt E. (2012) Alzheimer's disease and type 2 diabetes: Two diseases, one common link? *World J Biol Psychiatry*. 2012 Feb 14. IN PRESS

Participated in	**Author-Initials,** Responsibility decreasing from left to right					
Study Design	ED	PR	JB			
Data Collection	**JB**	AKW	JK			
Data-Analysis and Interpretation	**JB**	AKW	JK			
Manuscripte writing	**JB**	ED	PR			

I confirm that I have obtained permission from the co-authors see signature below:

C.M. Monoranu	
A.K. Wagner	
J. Kolter	
P. Riederer	
E. Grünblatt	

Publication: Bartl J., Meyer A., Brendler S., Riederer P., Grünblatt E.(2012) Different effects of soluble and aggregated Amyloid β_{42} on gene/ protein expression and enzyme activity involved in insulin and APP pathways *submitted to journal of neural transmission*						
Participated in	**Author-Initials,** Responsibility decreasing from left to right					
Study Design	**JB**	ED				
Data Collection	AM	SB	**JB**			
Data-Analysis and Interpretation	**JB**	ED	PR			
Manuscripte writing	**JB**	ED	PR			

I confirm that I have obtained permission from the co-authors signature below:

A. Meyer	
S. Brendler	
P. Riederer	
E. Grünblatt	

I also confirm my primary supervisor's acceptance.

Doctoral Researcher's Name Date Place Signature

Altern ist ein hochinteressanter Vorgang:

Man denkt und denkt und denkt –

plötzlich kann man sich an nichts mehr erinnern.

(Ephraim Kishon, Israelischer Schriftsteller)

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES 11
ABBREVATION INDEX 12
ZUSAMMENFASSUNG 14
SUMMARY 16
INTRODUCTION 18
GENERAL INTRODUCTION 18
EARLY AND LATE ONSET OF ALZHEIMER`S DISEASE 19
AMYLOID β CASCADE HYPOTHESIS 20
NEUROTOXICITY OF DIFFERENT AMYLOID β SPECIES 22
ALZHEIMER'S DISEASE=TYPE 3 DIABETES? 25
Role of peroxisome proliferator-activated receptor γ 28
THESIS OUTLINE 30
AIMS AND QUESTIONS 30
OVERVIEW OF MANUSCRIPTS 32
ABSTRACT MANUSCRIPT I 32
ABSTRACT MANUSCRIPT II 33
ABSTRACT MANUSCRIPT III 34
MANUSCRIPT I 36
MANUSCRIPT II 46
MANUSCRIPT III 60
DETAILED METHODS 82
MANUSCRIPT I 82
MANUSCRIPT II 85
MANUSCRIPT III 87
DISCUSSION 90
GENERAL DISCUSSION 90
Insulin degrading enzyme gene- a link of Alzheimer's disease with type 2 diabetes mellitus? 90
Common histopathological features of Alzheimer's disease and type 2 diabetes mellitus 92
Aggregation form of amyloid $β_{42}$ influences gene/ protein expression 94
OUTLOOK 96
ACKNOWLEDGEMENT 98
REFERENCES 100
CURRICULUM VITAE 106

LIST OF FIGURES AND TABLES

Figure 1:Braak stages define the distribution of neurofibrillary tangle (NFT) pathology in brains of Alzheimer's disease patients. 19

Figure 2:Electron microscope pictures of soluble and aggregated Aβ42 peptides. 22

Figure 3:Insulin signalling pathway. 24

Figure 4: Brain insulin resistance and amyloid precursor protein (APP) Aβ-mediated neurotoxicity.. 27

Figure 5: negative feedback loop between insulin and insulin receptor expression 93

Table 1: Age related plaque score according to the Consortium to Establish a Registry for Alzheimer's disease.. 18

Table 2: Demographic information of the VITA study cohort.. 83

Table 3: Demographic data of postmortem brains 85

ABBREVATION INDEX

A
ACTB = actin beta
AD = Alzheimer’s disease
AIC = Akaike Information Criterion
AID = APP intracellular domain
AKT = protein kinase B
ANOVA = analysis of variance
Apo = apolipoprotein
APP = amyloid precursor protein
Aβ = amyloid beta

B
BACE = β secretase
BBB = blood brain barrier
BMI = body mass index
BS = blocking solution
BSA = bovine serum albumin

C
°C = celsius
Ca = Calcium
CERAD = Consortium to Establish a Registry for AD
cDNA = copy DNA
CO_2 = carbon dioxide

D
DMEM = dulbecco's modified eagle's medium
DNA = deoxyribonucleic acid

E
ELISA = enzyme linked immunsorbent assay
ECL = enhanced chemiluminescence

F
FAD = familial AD

G
g = gramm
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GSK = glycogen synthase kinase

H
H_2O_2 = hydrogen peroxide
HCL = hydrochlorid acid
HRP = horse redish peroxidase
HWE = Hardy Weinberg equilibrium

I
icv = intracerebroventricular
IDE = insulin degrading enzyme
IGF = insulin growth factor
IL = interleukin
IR = insulin receptor
IRS = insulin receptor substrate

K
KCL = potassium chloride
kD = kilo Dalton

L
l = liter
LOAD = late onset AD

M
MAO = monoamin oxidase
ml = millilitre
mM = millimolar

N
nAChR = nicotin actelycholin receptors
NFT = neurofibrillary tangles
NINCDS-ADRDA = National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
nm = nanometer
NMDAR = glutamate receptors
NSE = neurospecific enolase

O
OS = oxidative stress

P
PCR = polymerase chain reaction
PI3K = phosphoinositide 3-kinases
PPAR = peroxisome proliferator-activated receptors
PPIA = peptidylprolyl isomerase A
PS = presenilin

Q
QRT-PCR = real time reverse transcriptase PCR

R
RNA = ribonucleic acid
RPL13A = ribosomal protein L13a
ROS = reactive oxidative species

S
sAPPα =soluble N-terminal APP fragment-α
APP
SD = standard deviation
SNP = single nucleotide polymorphism
STZ = streptozotocin

T
T1DM = type 1 diabetes mellitus
T2DM = type 2 diabetes mellitus
TBS = tris buffered saline
TDZ = thiazolidinediones
TNF = tumor necrosis factor
TOMM = a transporter of proteins across the
mitochondrial membrane

U
UTR = untranslated region

V
VITA = Vienna Transdanube Aging

W
WB = western blot

ZUSAMMENFASSUNG

Die neurodegenerative Erkrankung Alzheimer Demenz (AD) ist für etwa 60% der weltweit 35 Millionen Demenz Patienten ursächlich. Die aktuelle Forschung konzentriert sich hierbei auf Assoziationen mit anderen Erkrankungen wie Diabetes Typ 2 (T2DM), potentielle genetische Marker, spezifische Signaltransduktionswege im Gehirn und mögliche Modifizierung von Proteinen, da weder die Pathogenese noch die Ätiologie von AD vollständig geklärt ist. Im Jahr 1999 rückte durch die so genannte "Rotterdam-Studie" eine mögliche Verbindung zwischen T2DM und AD in den besonderen Fokus der Wissenschaft, da die Studie darauf hinweist, dass T2DM das Risiko eine AD zu entwickeln verdoppeln kann. In der Zwischenzeit ist bekannt, dass die Prävalenz an einer AD zu erkranken bei Patienten mit T2DM 30% beträgt. Zusätzlich zeigten Medikamente, die häufig zur Behandlung von T2DM eingesetzt werden, wie PPARγ (Peroxisom-Proliferator-aktivierte Rezeptoren gamma) Agonisten, eine Verbesserung der kognitiven Leistung bei Patienten mit einem frühen Stadium der AD. Daher ist es wichtig, nicht nur eine mögliche Verbindung zwischen diesen Krankheiten zu untersuchen, sondern auch die Insulin-Signalwege im Gehirn von AD Patienten näher zu betrachten. Um dieses komplexe Thema in weiteren Details zu untersuchen und zusätzliche Verbindungen zwischen T2DM und AD aufzuzeigen, verwendet die vorliegende Studie mehrere biologische Grundlagenmethoden, um die Frage zu klären: "Ist ein beeinträchtigter zerebraler Insulin-Signalweg entscheidend für die Entwicklung einer AD?"

Die in dieser Arbeit verwendete Methoden waren i) eine Analyse von Einzel-Nukleotid-Polymorphismen (SNP) des *Insulin-abbauende Enzym* (*IDE*) Gens in Bezug auf das Risiko eine AD und/oder T2DM zu entwickeln; ii) post-mortem histochemische Untersuchungen des Gehirngewebes von Patienten mit nur AD, mit AD und T2DM, und mit nur T2DM verglichen mit einer altersangepassten Kontrollgruppe; und iii) Untersuchungen neurobiologischer Signalwege und Gen-/Protein-Expressions Veränderung einer humanen Neuroblastoma Zelllinie nach Behandlung mit Amyloid β (Aβ) Peptiden.

Nach der Analyse der *IDE*-SNPs in der ausgewählten VITA (*Vienna Transdanube Aging*) Kohorte wurden krankheitsspezifische Effekte entdeckt. Der *Upstream*-Polymorphismus (IDE2) minderte das Risiko an einer AD zu erkranken, während der *downstream* gelegene Polymorphismus (IDE7) das Risiko T2DM zu bekommen, erhöhte. Basierend auf den SNP Ergebnissen, beschreibt die vorliegende Studie ein Modell, das Variationen innerhalb des *IDE* Promotors und/oder in untranslatierten Regionen unterschiedliche Auswirkungen auf die *IDE*

Expression haben können und somit potentiell Auswirkungen auf die Entwicklung von AD und T2DM haben können.

Darüber hinaus konnte die menschliche post-mortem Studie zeigen, dass sowohl AD als auch T2DM Patienten eine signifikant geringere Dichte der Insulin-Rezeptoren (IR) im Hippokampus hatten, während eine signifikant erhöhte Dichte von inaktiven phosphorylierten PPARγ bei allen Patientengruppen detektiert werden konnte. Die vorliegende post-mortem Studie konnte zwar gemeinsame histologische Merkmale von AD und T2DM aufzeigen, jedoch keine direkte Verbindung der beiden Erkrankungen nachweisen.

Obwohl AD heutzutage nicht mehr nur noch durch die Amyloid-haltigen Plaqueablagerungen und durch die hyperphosphorylierten Tau Proteine gekennzeichnet ist, spielt das übermäßige Vorhandensein von $A\beta_{42}$ in den Gehirnregionen von AD Patienten eine entscheidende Schlüsselrolle. Bis dato ist es immer noch nicht vollständig geklärt, welche physikalische Form von $Aß_{42}$ verantwortlich für eine Entwicklung von AD ist. Die vorliegende Arbeit untersuchte, welche Auswirkungen die Aggregatszustände von $Aß_{42}$ auf Gene und Proteine des Insulin-Signalweges und auf die Amyloid-Kaskade haben. Es konnte gezeigt werden, dass die oligomere Variante von $Aß_{42}$ speziell die Gen- und Proteinexpression von Glykogen-Synthase Kinase (GSK) 3β als auch ihre Enzymaktivität deutlich erhöht hatte, jedoch im Gegenzug die IR Gen- und Proteinexpression stark gehemmt hatte. Zusätzlich wurde die Wirkung von $Aß_{42}$ auf die Monoamin Oxidase-B (MAO-B) untersucht. Es wurde ein Effekt beider untersuchten aggregierten Formen von $Aß_{42}$ auf die Enzymaktivität entdeckt. Jedoch führte hier die fibrilläre Variante zu einer deutlich erhöhten Aktivität von MAO-B, während die oligomere Variante die Enzymaktivität inhibiert. Frühere Studien konnten bereits eine Beteiligung von erhöhter MAO-B-Aktivität in AD nachweisen, aber die vorliegende Arbeit zeigt erstmals eine direkte Verbindung zwischen den Aggregatzuständen von $Aß_{42}$ auf die Enzymaktivität auf.

Abschließend können die Ergebnisse der vorliegenden Arbeit zu folgenden Schlussfolgerungen zusammengefasst werden:

Obwohl AD und T2DM bis zu einem gewissen Grad gemeinsame Merkmale aufzeigen, fehlt es an einer direkten Verbindung, und somit sollten die Krankheiten weiterhin eher unabhängig als miteinander verbunden betrachtet werden. Jedoch scheint die Beeinträchtigung des zerebralen Insulin Signalweges ein weiteres gefestigtes Merkmal von AD zu sein.

SUMMARY

The neurodegenerative disorder Alzheimer's disease (AD) is the cause of approximately 60% of the world's 35 million patients suffering from dementia. Current research focuses here are on association with other diseases such as diabetes type 2 (T2DM), possible genetic markers, specific signal transduction pathways within the brain and potential protein modification, because the pathogenesis and etiology of AD are still not fully understood. Specifically association of T2DM with AD came to the focus with the so-called "Rotterdam study" in 1999, indicating that T2DM doubles the risk of developing AD. In the meantime, it is known that the prevalence rate in patients with T2DM is 30%. Drugs commonly used in the treatment of T2DM such as peroxisome proliferator-activated receptors gamma (PPARγ) agonists show improvement of the cognitive abilities in patients with early stage of dementia, with potential therapeutically relevance. Therefore it is important not only to investigate a link between these diseases, but also to investigate the insulin signaling pathway in the brain of AD patients. In order to investigate this complex issue in more details and demonstrate additional links between T2DM and AD, the present study used several basic biological methods to clarify the question: "Is impaired insulin signaling pathway within the brain crucial for the development of AD?" from several points of view. The methods used in this work have been i) an analysis of single nucleotide (SNP) polymorphism of the *insulin-degrading enzyme* gene (*IDE*) in relation to risk of AD and / or of T2DM, ii) post-mortem histochemical studies of brain tissue of patients with only AD, with AD combined with T2DM and with only T2DM compared with an age-matched control group, and iii.) investigations of neurochemical pathways and gene/protein expression changes of a human cell culture as a consequences of amyloid β (Aβ) treatment.

After analysis of the *IDE* SNP polymorphism in the selected VITA (Vienna Trans Danube Aging) cohort disease-specific effects were discovered. The upstream polymorphism (IDE2) was found to influence AD risk in a protective manner, while the downstream polymorphism (IDE7) modified the T2DM risk. Based on the SNP results, the presented study delineate the model that *IDE* promoter and 3' untranslated region/downstream variation can have different effects on *IDE* expression, maybe a relevant endophenotype with disorder-specific effects on AD and T2DM susceptibility.

Furthermore, the human post-mortem studies could show that both AD as well as T2DM patients had a significantly lower density of the insulin receptor (IR) in the hippocampus, whereas a significantly increased density of inactive phosphorylated PPARγ has been found

and this persisted even in patients with both diseases. Summarizing the histological study, it was possible to reveal common histological features of AD and T2DM, but no direct connection between the two diseases.

Although AD is nowadays not only characterized by amyloid-containing plaque deposits and by the hyperphosphorylation of tau protein, the excessive $A\beta_{42}$ presence in the brains of AD patients is still playing a key role. Up to date it is still not entirely clear which physical form of $A\beta_{42}$ is responsible for the development of AD. The present work investigated, what impact has the state of aggregation of $A\beta_{42}$ on genes and proteins of the insulin signaling pathway and the amyloid cascade. It could be shown that the oligomeric variant enhanced specifically the gene and protein expression of glycogen synthase kinase (GSK) 3β and also the enzyme activity was significantly increased, but has in turn strongly inhibited the IR gene and protein expression. Additionally, the effect of $A\beta_{42}$ on monoamine oxidase B (MAO-B) was examined. An effect of both aggregated forms of $A\beta_{42}$ had on enzyme activity was discovered. However, the fibrillar variants led to significantly increased activity of MAO-B while the oligomeric variants inhibited the enzyme activity. Several previous studies have demonstrated the involvement of increased MAO-B activity in AD, but the present work provides for the first time a direct link between the states of aggregation of $A\beta_{42}$ to enzyme activity.

Finally the results of the presented thesis can be summarized to following conclusion: Although AD and T2DM sharing some degrees of common features, still there is a lack of direct association, and therefore the diseases must be considered more independent rather than linked. But the impaired cerebral insulin signaling pathway seems to be another manifested hallmark of AD.

INTRODUCTION

GENERAL INTRODUCTION

Alois Alzheimer (*14th of June, 1864; † 19th of December, 1915) described already 1906 in Tübingen the first symptoms of the progressive neurode-generative disorder "Alzheimer's disease (AD)" according to his observations of his patient Auguste Deter [1]. The patient showed progressive loss of memory, deteriorating orientation in time and space, became bedridden and incontinent, and died four and half year after the first symptoms. At post-mortem examination, Alois Alzheimer noted the presence of abnormal fibrous inclusions within the perikaryal cytoplasm of pyramidal neurons and neuritic plaques with a central core of amyloid-β (Aβ) peptides. Up to date neuropathological diagnosis of AD is based on the frequency of neuritic plaques in neocortex determined according to the Consortium to Establish a Registry for AD (CERAD) criteria [2] (table 1) while the Braak scores [3] define the distribution of neurofibrillary tangle (NFT) ,which are composed of hyperphosphorylated forms of the microtubule-associated protein tau, pathology in the entorhinal cortex, hippocampus, and neocortex (figure 1).

Table 1: Age related plaque score according to the Consortium to Establish a Registry for Alzheimer's disease

Age of patient at death (years)	Frequency of plaques			
	none	*sparse*	*moderate*	*frequent*
<50	0	C	C	C
50-75	0	B	C	C
>75	0	A	B	C

An age related plaque score is determined using patient's age along with plaque frequency in the most heavily affected brain section like frontal, temporal and/or parietal cortex. 0= No histologic evidence of Alzheimer's disease (AD); A= Histologic findings are uncertain evidence of AD; B= Histologic findings suggest the diagnosis of AD; C= Histologic findings indicate the diagnosis of AD [2]

The progressive deposition of NFT has been divided into six stages and AD is usually diagnosed when the neuropathological changes have reached stage III to VI. In the transentorhinal stage (Braak stages I and II), the deposition is still restricted to the entorhinal cortex and the parahippocampal cortex, which play a role in the formation of memories and the identification of odors Disturbed function in these areas probably underlies early clinical changes of AD, including delayed memory recall and odor identification [4], that disrupts daily life, challenges in planning or solving problems, difficulty completing familiar tasks at home,

at work, or at leisure, confusion in time or place, trouble understanding visual images and spatial relationships, new problems with words in speaking or writing, misplacing things and losing the ability to retrace steps, decreased or poor judgment, withdrawal from work or social activities, changes in mood and personality (reviewed in [5]). Although memory impairment is the earliest cognitive change in AD, distinguishing early disease from normal aging can be difficult. With disease progression, signs and symptoms include confusion, irritability, and aggression, mood swings, language breakdown, loss of long-term memory, and general withdrawal decline. The mean life expectancy following diagnosis is approximately 7 years [6].

.

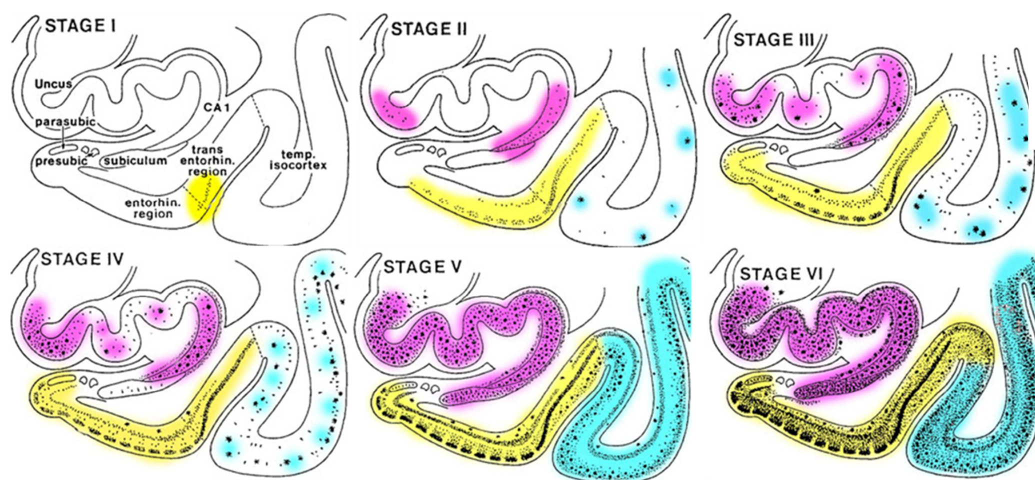


Figure 1: Braak stages define the distribution of neurofibrillary tangle (NFT) pathology in brains of Alzheimer's disease patients. At Braak stage I, the NFT have begun to form in the transitional entorhinal region; at Braak stage II, NFT are causing cell death (yellow) in the transitional entorhinal region and aggregating in the hippocampus (pink) and in the cortex (blue). In stages III and IV (limbic stages), the NFT are already in the hippocampal formation and in other centers of the limbic system before. Braak stages V and VI (isocorticale stages) are the final stages of AD, where the presence of NFT already in the isocortex. Images according to [3]

EARLY AND LATE ONSET OF ALZHEIMER`S DISEASE

Two types of AD are needed to be distinguished: early onset AD, also called familial AD (FAD) is mostly genetically based, while the late onset AD (LOAD) is a sporadic form and probably involved both polygenetic and environmental factors. FAD accounts for approximately 5% of all AD cases and leads to an earlier begin of the disease with around 45 years of age. Genes involved in FAD are coding for amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2). Mutations in the PS1 and PS2 genes account for as much as 50% of all FAD cases. More than 30 mutations in the APP gene and more than 180

mutations in PS1 and PS2 genes have been already identified. Mutations in all three genes were shown to increase the production and aggregation of Aβ (reviewed in [7]). Testing of asymptomatic adults who are at risk for FAD caused by mutations in the APP, PS1 or PS2 genes is available clinically. However, genetic testing results for at-risk asymptomatic adults can only be interpreted after the disease-causing mutation has first been identified in the affected family member. It should be emphasized that testing of asymptomatic at-risk individuals with nonspecific or equivocal symptoms is predictive but is not diagnostic. In addition, obtaining results from genetic testing can affect an individual's personal relationships as well as their emotional well-being, and it may even cause depression[8]. Clinically and histopathologically, FAD cannot be discriminated from LOAD. The majority of AD patients (~ 95%) have LOAD and the disease usually develops after 65 years of age. Expression of the apolipoprotein E allele ε4 (ApoEε4) is one of the risk factors identified for LOAD and this risk is highest for individuals that carry two ApoEε4 alleles (ε4/ε4 genotypes). The ε4/ε4 genotype is uncommon, occurring in about 1% of normal Caucasian controls. In contrast, the ε4/ε4 genotype occurs in nearly 19% of LOAD populations. Women with an *ApoE*ε4/ε4 genotype have a 45% probability of developing AD by the age of 73, whereas men have a 25% risk of developing AD by that age. AD risk is also lower for individuals with only one *ApoE*ε4 allele (by age 87) or no *ApoE*ε4 allele (by age 95) (reviewed in [9]). The role of ApoEε4 in LOAD pathogenesis is not fully elucidated, but it has been suggested that ApoEε4 is important in trafficking of Aβ peptide. Recently discovered proteins encoded by risk genes of LOAD are apolipoprotein J (clusterin, an Aβ peptide chaperone), TOMM40 (a transporter of proteins across the mitochondrial membrane) and a sortilin-related receptor, which functions to partition APP away from β-secretase and γ-secretase, which are the main cleavage enzymes of APP [10]. Additional factors, such as hypertension, diabetes mellitus, hyperlipidemia, hyperhomocysteinemia, coronary and peripheral artery diseases, alcohol, smoking, obesity, levels of physical or mental activity, levels of education, and environmental exposures have been investigated to identify risk factors for LOAD (reviewed in [11]). The main focus of this on hand dissertation based on several published manuscripts lay on LOAD and so the following chapters are concentrating especially on this form of AD.

AMYLOID β CASCADE HYPOTHESIS

As mentioned before the hallmarks of AD are NFTs and accumulation deposits of Aβ peptides, but the latter being the focus of this thesis. The Aβ cascade hypothesis affirms some

major abnormality of processing of the APP that causes an overproduction of the $A\beta_{42}$ fragment with a molecular weight of 4kDa. APP is a transmembrane protein expressed in several different tissues but is mainly concentrated in synapses of neurons. The physiologic function of APP remains undefined, but immunohistochemical studies have localized APP activity to the plasma membrane, the trans-Golgi network, the endoplasmic reticulum, and the endosomal, lysosomal, and mitochondrial membranes [12] and it has been implicated as a regulator of synapse formation, neural plasticity [13] and iron export [14]. It can be processed through the so called "amyloidogenic" or the "non-amyloidogenic" pathway. In this process, three enzyme complexes are involved; α-, β- and γ-secretase. The non-amyloidogenic pathway cleaves APP through α- and γ- secretases and results in a p_3 peptide, in a soluble N-terminal APP fragment-α (sAPPα) and in an APP intracellular domain (AID). It is suspected that sAPPα has a neuroprotective function [15], AID seems to regulate phosphoinositide-mediated calcium signaling pathway [16], and the function of p_3 remains unclear. The amyloidogenic pathway is the cleavage of APP by β-secretase (BACE) instead of α-secretase, and produces the Aβ peptides, but also sAPPβ and AID. There are several possible lengths of Aβ peptides, since the C-terminus of the γ-secretase can cut at different positions. The length of these Aβ peptides can vary between 39-42 amino acids, wherein the $A\beta_{40}$ peptides are soluble and the $A\beta_{42}$, are insoluble and can accumulate. Because of its role in Aβ production, BACE is a possible target for potentially new drugs inhibiting Aβ peptides production [17]. However, the mechanisms that contribute to especially abnormal $A\beta_{42}$ accumulation of AD are not fully understood, both increased production and decreased degradation have been observed [18]. At present, it is not understood whether these peptides act via receptors or whether membrane binding alone leads to synaptic and neuronal degeneration. Membrane interaction of $A\beta_{42}$ peptides could occur via hydrophobic carboxy-terminal domain or by electrostatic interactions mediated by the charged amino acids in the amino-terminal domain. $A\beta_{42}$ may bind to the cell membrane to form channels or pores that disrupt ion homeostasis, hence leading to neuronal dysfunction (reviewed in [19]). It is know that it can bind to nicotin acetylcholine receptors (nAChR), glutamate receptors (NMDAR) and calcium $(Ca)^{2+}$ ion channels (e.g. voltage-dependent calcium channels, transient receptor potential cation channels) and causes Ca^{2+} influx and elevates the levels of potentially toxic reactive oxygen species (ROS) in an NMDAR-dependent manner. Several reports of the effect of $A\beta_{42}$ on nAChRs are conflicting. Some studies have reported that $A\beta_{42}$ activates nAChRs, while others indicate that $A\beta_{42}$ inhibits nAChRs. For example, physiological levels of $A\beta_{42}$ can activate

while toxic levels inhibit presynaptic nAChR and evoke changes in presynaptic Ca^{2+} levels in rat hippocampus and neocortex. Immunohistochemical studies on human sporadic AD brains show that $A\beta_{42}$ and nAChR, are both present in neuritic plaques and co-localize in individual cortical neurons suggesting that $A\beta_{42}$ could be tightly associated with nAChR (reviewed in [20]). Loss of synaptic terminals or neuronal degeneration could cause the associated decline in cognitive functions that characterizes AD. Whether the neurotoxic and synaptotoxic actions of $A\beta_{42}$ are separate activities or whether they share common mechanisms is not known.

NEUROTOXICITY OF DIFFERENT AMYLOID β SPECIES

$A\beta_{42}$ are able to adopt many differently shaped aggregates including amyloid fibrils [21] as well as nonfibrillar aggregates that are also termed $A\beta_{42}$ "oligomers" [22] (figure 2).

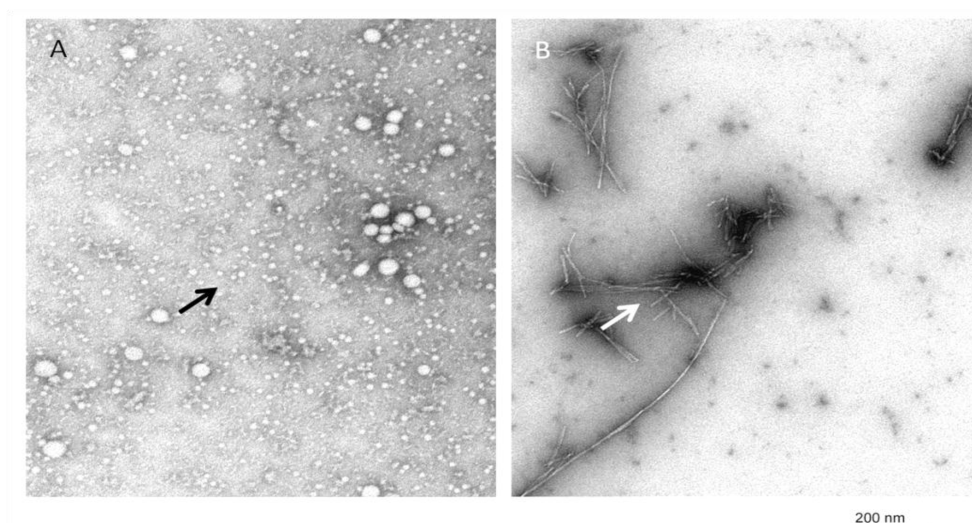


Figure 2: Electron microscope pictures of soluble and aggregated $A\beta_{42}$ peptides. 5µl of samples were spotted on a glow discharged, carbon-coated grid and incubated for 5 min, washed with distilled water, fixed with 2.5% glutaraldehyde, stained with a 1% (w/v) aqueous uranyl acetate solution, and examined using a transmission electron microscope. 20.000 fold magnification. A. black arrow points to soluble $A\beta_{42}$ peptides; B. white arrow points to aggregated $A\beta_{42}$ peptides (own images; for details see Manuscript III).

It is not well established which $A\beta_{42}$ state is most responsible for AD or why. Recent evidence strongly implicates $A\beta_{42}$ oligomers as the proximal pathogenic trigger [23]. These oligomers are markedly elevated in the brain and cerebrospinal fluid of post-mortem AD patients and appear to play a critical role in the synaptic failure and memory deficits of early AD [24]. The importance of $A\beta_{42}$ oligomers is especially evident as they appear necessary and sufficient to alter long term potential *in vivo* [25] and *in vitro* [26] and as they appear to actually

reduce the density of synapses [27]. At the molecular level, different $A\beta_{42}$ aggregates act by increasing inward excitatory post-synaptic currents with membrane depolarization through the AMPA and NMDA channels [28], and they are also able to alter neuronal architecture [29], cause perturbations in axonal transport and even down-regulate cell surface levels of NMDA receptors [30]. $A\beta_{42}$ is able to bind to a variety of biomolecules, including lipids, proteins and proteoglycans, like integrins, or APP itself [31]. The binding of $A\beta_{42}$ to membrane lipids facilitates $A\beta_{42}$ aggregation, which in turn disturbs the structure and function of the membranes, such as membrane fluidity or the formation of ion channels and can hypothetically sparking a signal that leads to cell death or acting via intracellular initiated damage, e.g., death through a calcium mediated mechanism [32], or disturbing physiological ion exchange [33]. One of the most known and studied effects of $A\beta_{42}$ is, in fact, its ability to induce, and be induced by, oxidative stress (OS). In 2004, Riederer et al. already reviewed the link between OS and AD, especially regarding the increased monoamine oxidase B (MAO-B) activity [34]. MAO-B is a flavin containing enzyme localized in the outer mitochondrial membrane and is responsible for the oxidative deamination of neurotransmitters (norepinephrine, dopamine and serotonin) and exogenous amines. During its catalytic activity it produces hydrogen peroxide formed in the reaction, which is a possible source for oxidative stress, and inhibition of MAO-B has a neuroprotective effects for AD. Not only increased MAO-B activity is associated with AD, also other oxidative damage of biomacromolecules has been already described in the brains of AD patients: i.) DNA and RNA oxidation is marked by increased levels of 8-hydroxy-2-deoxyguanosine and 8-hydroxyguanosine; ii.) protein oxidation is marked by elevated levels of protein carbonyl and nitration of tyrosine residues, iii.) lipid peroxidation is marked by high levels of thiobarbituric acid-reactive substances, malondialdehyde, 4-hydroxy-2-nonenal, and isoprostanes and altered phospholipid composition; and iv.) modification to sugars is marked by increased glycation and glycoxidation (reviewed in [35]). Both amyloid deposits and soluble $A\beta_{42}$ seem to drive the accumulation of reactive oxidative species (ROS) [36]. Furthermore, $A\beta_{42}$ can strike the production of pro-inflammatory molecules, such as TNF-α and IL-1β, leading to microglial activation, production of an immune response, and to an enhanced production of APP and its processing to generate more $A\beta_{42}$ (reviewed in [37]). Additionally $A\beta_{42}$ oligomers interact with neuronal insulin receptor (IR) via binding to cause impairments of the receptor expression and function [38,39]. Normally, activation of IR leads to the phosphorylation of phosphoinositide-3 kinase (PI3K), which leads to an activation of AKT. PI3K has been identified as second

messenger system providing anti-apoptotic signal to various cell types [40] and also neuroprotection against extracellular $A\beta_{42}$ toxicity is dependent on PI3K pathway [41,42]. Once AKT is activated, it leads to the phosphorylation and consequently to the inactivation of glycogen synthase kinase (GSK) 3 [43] (figure 3):

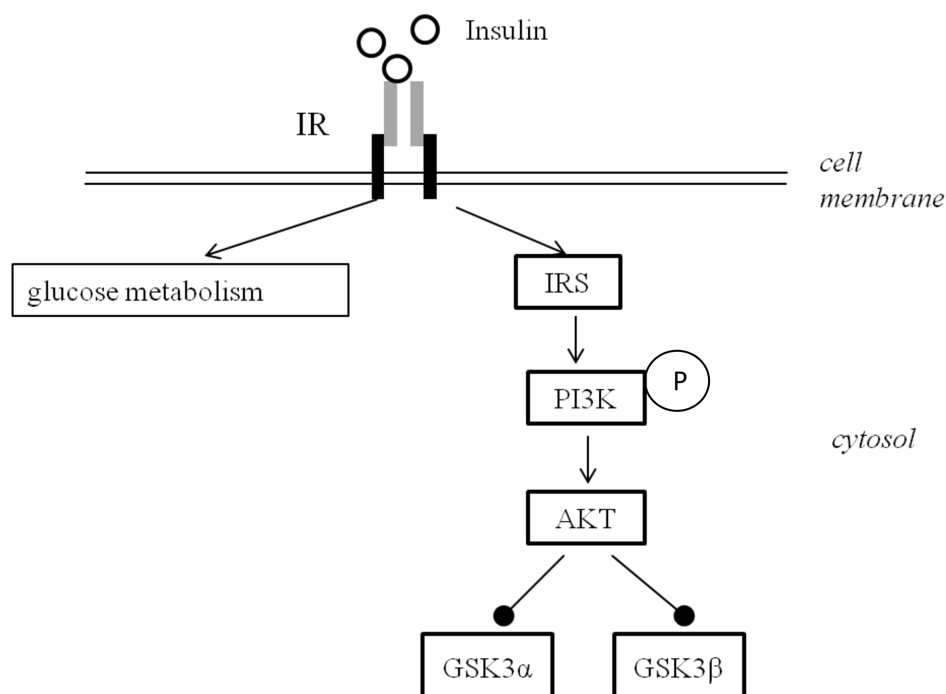


Figure 3: Insulin signalling pathway. Insulin activates the transmembran insulin receptor (IR) leading to the activation of intracellular insulin receptor substrate (IRS). Active IRS leads to phosphorylation of phosphoinositide-3 kinase (PI3K), which activates protein kinase B (AKT). Active AKT leads to the phosphorylation and consequently to the inactivation of glycogen synthase kinase (GSK) 3α and 3β. P = phosphorylated. → = activation; • = inhibition

There are two *GSK3* genes from which GSK3α and GSK3β are derived. GSK3α and GSK3β are ubiquitously expressed, constitutively active, proline-directed serine/threonine kinases involved in a variety of cellular processes including glycogen metabolism [44], gene transcription [45], apoptosis [46] and microtubule stability [47]. As seen in figure 3 GSK3 can be modulated by insulin and over-activity of GSK3 accounts for memory impairment, tau hyperphosphorylation, increased Aβ production and local plaque-associated microglial-mediated inflammatory responses; all of which are hallmark characteristics of AD (reviewed in [48]). Thus, although normal insulin and IR activity help to defend against the accumulation of toxic $A\beta_{42}$ oligomers, the IR themselves are vulnerable to oligomer-initiated dysfunction. Another interesting factor is, that the insulin degrading enzyme (IDE) apart from its primary target insulin, additionally degrades $A\beta_{40}$, $A\beta_{42}$ and the AID and to eliminate $A\beta_{42}$ neurotoxic

effects [49]. The first evidence that IDE might be involved in $A\beta_{42}$ degradation was found by Kurochkin and Goto in 1994, who demonstrated that purified rat IDE efficiently degrades synthetic $A\beta_{42}$ *in vitro* [50]. Subsequently, it was shown that an IDE-like activity from soluble and synaptic membrane fractions of human post-mortem tissue both degrade $A\beta_{42}$ peptides [51,52]; moreover, IDE doesn't distinguish between endogenous and synthetic $A\beta_{42}$ as substrate *in vitro* [53,54]. The link between $A\beta_{42}$, IR and IDE leads to the question: "Is an impairment of the insulin signalling pathway a potential trigger for AD?"

ALZHEIMER'S DISEASE=TYPE 3 DIABETES?

The association between type 2 diabetes mellitus (T2DM) and increased risk of dementia in the elderly is well investigated [55,56] and it started all with the groundbreaking "Rotterdam study" in the year 1999 [57]. They recruited 6,370 elderly subjects and studied them for around 2 years. 126 developed dementia and 89 of these were specifically diagnosed with AD. They detected that T2DM doubled the risk of a patient having dementia and patients on insulin had four times the risk. Starting from this study, multiple possible mechanisms for this association have been proposed, including the direct effects of hyperglycaemia, insulin resistance, and insulin-induced amyloid-peptide amyloidosis in the brain [58]. In the early stages of AD, cerebral glucose utilization is reduced by as much as 45%, and blood flow by ∼18% [59,60] and since it is known that insulin is also synthesised in the central nervous system, it may contribute to an underlying pathophysiological mechanism for AD. Insulin binds to IRs in the brain, most of which are located in the cerebral cortex, olfactory bulb, hippocampus, cerebellum, and hypothalamus and activates not only signaling pathways associated with learning and long-term memory; it also helps to regulate processes such as neuronal survival, energy metabolism, and plasticity. These processes are required for learning and memory. In addition to regulating blood sugar levels, insulin functions as a growth factor for all cells, including neurons in the brain. Thus, insulin resistance or lack of insulin, in addition to adversely affecting blood sugar levels, contributes to degenerative processes in the brain (reviewed in [61]). Peripheral insulin resistance, therefore, affects cognition. Furthermore the fact that insulin is a common substrate for most of the identified Aβ-degrading enzymes, such as IDE, has drawn attention of investigators to roles of insulin signaling in Aβ clearance. Increases in insulin levels frequently seen in insulin resistance may compete for these enzymes and thus contribute to Aβ accumulation [62]. The fact that extracellular $A\beta_{42}$ deposition is one of the hallmarks of AD made *IDE* a promising candidate gene involved in

disease susceptibility. Several studies associated linkage-peaks over the *IDE* region with LOAD [63], age of onset in familial AD [64] and high plasma $A\beta_{42}$ levels [65]. A recent study found that variants in the proximal *IDE* promoter increase transcription and thus provide a possible mechanism explaining the protective effect on AD susceptibility found for the examined polymorphisms [66]. Furthermore, validated linkage peaks with the metabolic syndrome T2DM were found in the same region [67-70]. Not only enzymes are involved of the cleavage of Aβ. Circulating insulin like growth factor 1 (IGF-1) has been reported to play a role in Aβ clearance probably via facilitating brain-blood barrier (BBB) transportation [71]. Additionally in the endothelium of the BBB and glial cells, insulin signaling is reported to regulate protein-protein interactions in an uptake cascade involving low density lipoprotein receptor-related protein and its ligands ApoE and α2-macroglobulin, a system known to bind and clear Aβ via endocytosis and/or vascular transport [72].

A summary of some of the earlier findings in AD, including the impaired glucose utilization, OS via mitochondrial dysfunction, reduced ATP production, and energy shortage, led to the hypothesis that these abnormalities were mediated by desensitization of the neuronal IR. Hoyer and colleagues were among the first to suggest that reduced levels of brain insulin may precipitate a cascade resulting in disturbances in cellular glucose, acetylcholine, cholesterol, and ATP levels, impaired membrane function, accumulation of amyloidogenic derivatives, and hyper-phosphorylation of tau, i.e. that AD may represent a brain form of T2DM [73,74]. The question: “Can AD be described as a kind of type 3 diabetes?” was coined in 2005 by Suzanne de la Monte and her team [75]. They analyzed 45 post-mortem brains of patients of varying Braak stages of AD neurodegeneration and found that insulin expression was inversely proportional to the Braak stage with an 80% decrease in the number of insulin receptors in AD patients compared to normal subjects with the ability of insulin to bind to the receptors was compromised. The expression of insulin, IGF-1 and -2 and their receptors mRNA was reduced. The team termed it type 3 diabetes because it includes elements of both types 1 and 2 diabetes, as there is both a decrease in the production of insulin and a resistance to IRs. The post-mortem studies inspired a rat model in which intracerebroventricular (icv) injection of streptozotocin (STZ) were conducted. STZ is a nitrosamide methylnitrosourea linked to D-glucose, and is a drug selectively toxic for insulin producing/secreting cells, as following systemically application. STZ enters the cells via the glucose transporter 2 (GLUT2), mainly localized in pancreatic b-cells (to a certain extent also in hepatocytes and absorptive epithelial cells of the intestine and kidney). Once STZ is metabolized, the N-

nitrosoureido is liberated and caused DNA damage and cell death through generation of ROS [76]. STZ icv did not cause a systemic diabetes mellitus [77,78] and is normally used in lower dosages than used for systematic application, but impairs brain glucose utilization oxidative metabolism, IR function, and spatial learning and memory. Moreover, icv-STZ treatments produced long-term and progressive deficits in learning, memory, cognition, behavior, and cerebral energy balance. Therefore, the icv-STZ model recapitulates many characteristic features of AD, like long-term and progressive deficits in learning, memory, cognition, behavior, cerebral energy balance and demonstrates a cause-effect relationship between insulin deficiency and resistance and AD-type neurodegeneration, like a Type 3 diabetes (reviewed in [79]). Figure 4 demonstrates a possible pathway of insulin, insulin resistance, OS, Aβ and T2DM involved in AD modified according to de la Monte (2012) [80]:

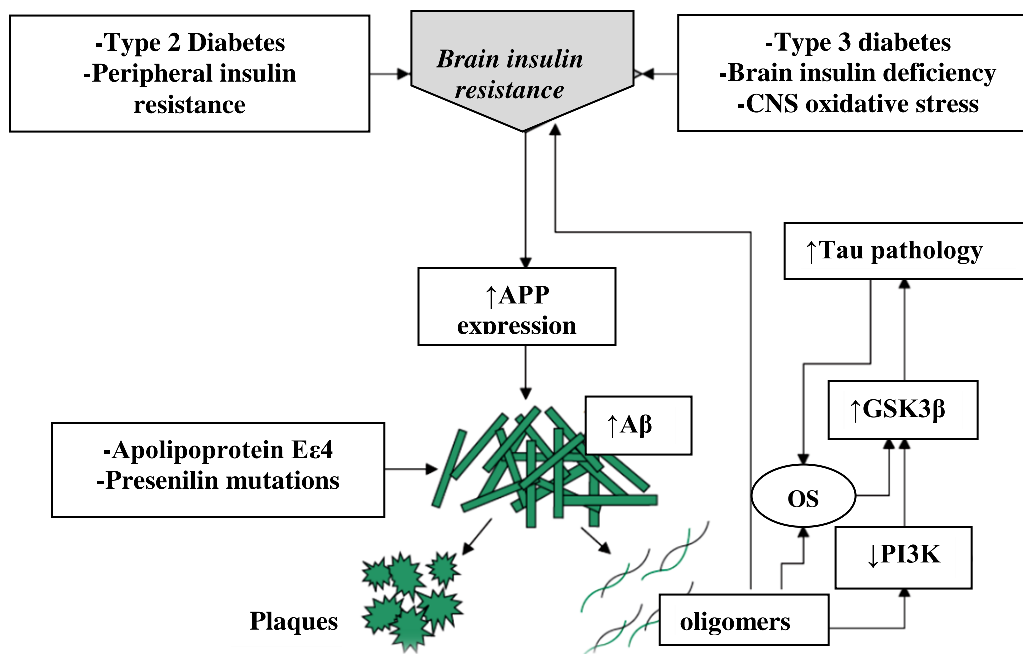


Figure 4: Brain insulin resistance and amyloid beta (Aβ)-mediated neurotoxicity. Brain insulin resistance caused by peripheral insulin resistance diseases or intrinsic/genetic processes, toxic exposures or environmental factors contributing to neurodegeneration promote neuroinflammation and increased expression of amyloid precursore protein (APP). Through the action of β and γ secretases, APP is cleaved to generate 40–42 kD Aβ peptides that aggregate and form insoluble fibrils and plaques, or oligomers. Aβ oligomers promote oxidative stress and activate kinases that lead to tau accumulation, hyperphosphorylation and eventual ubiquitination, misfolding and aggregation. Aβ can block insulin-receptor function and contribute to insulin resistance. Carriers of the apoliprotein Ee4 allele or presenilin mutations are predisposed to abnormal APP cleavage, and Aβ accumulation, aggregation and fibril formation, correlating with increased rates and familial occurrences of Alzheimer's disease. This scenario depicts a positive feedback or reverberating loop linking Aβ and oligomer accumulation/toxicity with brain insulin resistance, and vice versa. GSK3β = glycogen synthase kinase 3β; OS= oxidative Stress; PI3K= phosphoinositide-3-kinase; ↓ indicates decrease; ↑indicates increase. Modified according to [80]

Role of peroxisome proliferator-activated receptor γ

The nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) is a newly recognized potential therapeutic target for the treatment of AD. It is a member of a family of three (α/γ/δ) related nuclear receptors that act as whole body lipid sensors and each member functions to regulate a unique subset of genes responsible for lipid and energy metabolism [81]. The natural ligands of PPARγ include long-chain fatty acids, eicosanoids, oxidized lipoproteins and lipids, corresponding well to its function in regulating the metabolic response to dietary lipid intake. Thus, PPARγ has been targeted for drug development for the treatment of T2DM. Two thiazolidinedione (TZD) agonists of PPARγ, Actos™, (pioglitazone) and Avandia™ (rosiglitazone) are widely prescribed for this disease [82]. TDZ act as insulin sensitizers and reduce glucose, fatty acid, and insulin blood concentrations. 2005 Watson et al. [83] have reported the results of a small clinical study examining the effects of rosiglitazone in patients with mild AD. They found that 6 months of drug treatment resulted in enhanced memory and cognitive function compared to placebo-treated control patients. Afterwards the results of a phase II clinical trial, enrolling over 500 patients with mild to moderate AD were reported. These patients were treated with rosiglitazone (or placebo) for 6 months. Patients receiving rosiglitazone were found to have enhanced attention and memory, compared to those receiving placebo. Importantly, patients possessing an *ApoEε* allele did not respond to the therapy. A large phase III trial of rosiglitazone in AD patients is currently underway (reviewed in [82]). A possible mechanism of PPARγ agonist action comes from the fact that a consensus binding site for PPARγ was found in the *BACE* promoter. PPARγ activation by agonists such as TZD results in a decrease of *BACE* transcription, expression and activity [84]. Furthermore, lack of PPARγ led to an increase of *BACE* promoter activity [85], which suggested that PPARγ could be a repressor of *BACE*. PPARγ levels are decreased in AD brain, indicating that inflammatory events may decrease PPARγ transcription. Furthermore, *in vitro* experiments have shown that inflammatory cytokines and oxidative stress decrease PPARγ levels. Therefore, these findings suggest the existence of a down-regulation of PPARγ under inflammatory conditions, which would result in an increase in *BACE* transcription and Aβ generation [86]. Furthermore d'Abramo et al.[87] could indicate that PPARγ decreases the cellular content of APP without affecting its mRNA level, thus implying a post-transcriptional event, unlikely to be linked to the inhibition of the secretase pathways. Importantly, the evidence that PPARγ stimulates the ubiquitination of APP supports the fact that the Aβ-lowering effect of PPARγ is due to the proteasome-mediated degradation of APP. Additionally they could

show that PPARγ, by decreasing Aβ secretion, protects the cells against hydrogen peroxide (H_2O_2)-mediated necrosis. Thus, it seems that PPARγ agonist can represents new therapeutic target in treating AD.

THESIS OUTLINE

AIMS AND QUESTIONS

The possible association between T2DM and increased risk of AD in the elderly is in focus of the update AD research. Multiple possible mechanisms for this association have been proposed, including direct effects of hyperglycaemia, insulin resistance, and insulin-induced Aβ amyloidosis in the brain. Those observations are not without controversy, and a longitudinal survey revealed that although marginal diabetics had a significantly increased risk for future development of full diabetes, dementia, or AD, the risk effects were independent rather than linked. Thus, further investigations are necessary to delineate possible links.

Aim of this thesis was to use several molecular biological methods including analysis of SNP polymorphism, immunohistochemistry of post-mortem brain tissue as well as human cell culture model for investigations of biological pathways to ascertain this complex issue from several sides in order to get a more profound knowledge about basic principles underlying AD. The following main questions were addressed:

1. Do variations in the *IDE* gene, which is encoded for a cleavage enzyme for insulin and $A\beta_{42}$, have an impact on AD and/or T2DM susceptibility? (the corresponding results are summarized in Manuscript I, see below)

2. Which common histopatholigical features are sharing patient with solely AD, AD combined with T2DM, solely T2DM and is there a missing link? (the corresponding results are summarized in Manuscript II, see below)

3. Which influence does the aggregation form of $A\beta_{42}$ peptides have on gene expression, protein expression and enzyme activity involved in insulin and APP pathways? (the corresponding results are summarized in Manuscript III, see below)

OVERVIEW OF MANUSCRIPTS

ABSTRACT MANUSCRIPT I

Disorder-specific effects of polymorphisms at opposing ends of the Insulin Degrading Enzyme gene.

Jasmin Bartl, Claus-Jürgen Scholz, Margareta Hinterberger, Susanne Jungwirth, Ildiko Wichart, Michael K Rainer, Susanne Kneitz, Walter Danielczyk, Karl Heinz Tragl, Peter Fischer, Peter Riederer, Edna Grünblatt; *BMC Medical Genetics 2011, 12:151*

Abstract

Background: Insulin-degrading enzyme (IDE) is the ubiquitously expressed enzyme responsible for insulin and amyloid beta (Aβ) degradation. IDE gene is located on chromosome region 10q23-q25 and exhibits a well-replicated peak of linkage with Type 2 diabetes mellitus (T2DM). Several genetic association studies examined IDE gene as a susceptibility gene for Alzheimer's disease (AD), however with controversial results.

Methods: We examined associations of three IDE polymorphisms (IDE2, rs4646953; IDE7, rs2251101 and IDE9, rs1887922) with AD, $A\beta_{42}$ plasma level and T2DM risk in the longitudinal Vienna Transdanube Aging (VITA) study cohort.

Results: The upstream polymorphism IDE2 was found to influence AD risk and to trigger the $A\beta_{42}$ plasma level, whereas the downstream polymorphism IDE7 modified the T2DM risk; no associations were found for the intronic variant IDE9.

Conclusions: Based on our SNP and haplotype results, we delineate the model that IDE promoter and 3' untranslated region/downstream variation may have different effects on IDE expression, presumably a relevant endophenotype with disorder-specific effects on AD and T2DM susceptibility.

ABSTRACT MANUSCRIPT II

Alzheimer's disease and Type 2 Diabetes: two diseases, one common link?

Bartl Jasmin, Monoranu Camelia-Maria, Wagner Anne-Kristin, Kolter Jann, Riederer Peter, Grünblatt Edna; *The World Journal of Biological Psychiatry, 2012; 00: 1–8*

Abstract

Objectives: Although Alzheimer's disease (AD) is the most common form of dementia in the elderly, its aetiology remains mostly unknown. A potential pathophysiological mechanism for AD arises from the knowledge that insulin is also synthesized independently in the central nervous system and is involved in the regulation of memory formation. AD may represent a brain-specific form of insulin resistance.

Methods: We used immunohistochemistry to investigate the numbers of cells expressing insulin receptor β-subunit (IRβ) and phosphorylated PPARγ (PPARγ(p)) in human post-mortem tissue from patients with AD; AD combined with type 2 diabetes mellitus (T2DM); just T2DM, and from aged-matched controls. These numbers were evaluated in frontal cortex and in dorsal/ ventral parts of the hippocampus.

Results: We observed significantly lower numbers of IRβ positive cells in AD cases compared to all other groups in all investigated brain regions. Also significantly more PPARγ(p) positive cells occurred in each patient group compared to control.

Conclusions: T2DM and AD may not be directly linked, but may share common histological features including lower numbers of IRβ positive cells and higher numbers of PPARγ(p) positive cells in all investigated brain regions. These observations may at least partially explain the increased frequency of AD in elderly diabetic patients.

ABSTRACT MANUSCRIPT III

Different effects of soluble and aggregated Amyloid β_{42} on gene/ protein expression and enzyme activity involved in insulin and APP pathways

Jasmin Bartl, Andrea Meyer, Svenja Brendler, Peter Riederer, Edna Grünblatt

under Revision at Journal of Neural Transmission

Abstract

Although Alzheimer's dementia (AD) is not anymore characterised just by the accumulation and deposition of amyloid beta (Aβ) peptides and hyperphosphorylation of tau proteins within the brain, excessive $A\beta_{42}$ deposition is still considered to play a major role in AD. Aβ are able to adopt many differently aggregate forms, including amyloid fibrils as well as nonfibrillar structures (soluble $A\beta_{42}$ oligomers). It is not well established which $A\beta_{42}$ state is most responsible for AD or why. We wanted to verify, which effects $A\beta_{42}$ oligomers and aggregated peptides have on gene expression, protein level and enzyme activity of insulin and amyloid precursor protein (APP) pathways *in vitro.* Human neuroblastoma cells (SH-SY5Y) were treated with varying concentrations of soluble and aggregated $A\beta_{42}$. Treatment effects on β-secretase (BACE), glycogen synthase kinase 3α (GSK3α), glycogen synthase kinase 3β (GSK3β), phosphatidylinisitol-3 kinase (PI-3K), insulin degrading enzyme (IDE), insulin receptor substrate 1 (IRS1), insulin receptor (INSR) and monoamine oxidase B (MAO-B) was investigated via quantitative-PCR, western blot, ELISA and enzyme activity assay. We could find different effects of soluble and aggregated peptides especially on gene/ protein expression of GSK3β and INSR and on GSK3β and MAO-B activity. Soluble peptides showed significant effects leading to increased gene expression and protein amount of GSK3β and to decreased level of gene and protein expression of INSR. MAO-B activity was enhanced after treatment with aggregated peptides and strongly inhibited after soluble $A\beta_{42}$ treatment. Our data might provide insights into selective effects of specific forms of $A\beta_{42}$ aggregates in AD.

MANUSCRIPT I

BMC Medical Genetics

RESEARCH ARTICLE Open Access

Disorder-specific effects of polymorphisms at opposing ends of the *Insulin Degrading Enzyme* gene

Jasmin Bartl[1*†], Claus-Jürgen Scholz[2†], Margareta Hinterberger[3], Susanne Jungwirth[3], Ildiko Wichart[3], Michael K Rainer[3], Susanne Kneitz[2], Walter Danielczyk[3], Karl H Tragl[3], Peter Fischer[3,4], Peter Riederer[1,3] and Edna Grünblatt[1,3,5]

Abstract

Background: Insulin-degrading enzyme (IDE) is the ubiquitously expressed enzyme responsible for insulin and amyloid beta (Aβ) degradation. *IDE* gene is located on chromosome region 10q23-q25 and exhibits a well-replicated peak of linkage with Type 2 diabetes mellitus (T2DM). Several genetic association studies examined *IDE* gene as a susceptibility gene for Alzheimer's disease (AD), however with controversial results.

Methods: We examined associations of three *IDE* polymorphisms (IDE2, rs4646953; IDE7, rs2251101 and IDE9, rs1887922) with AD, $A\beta_{42}$ plasma level and T2DM risk in the longitudinal Vienna Transdanube Aging (VITA) study cohort.

Results: The upstream polymorphism IDE2 was found to influence AD risk and to trigger the $A\beta_{42}$ plasma level, whereas the downstream polymorphism IDE7 modified the T2DM risk; no associations were found for the intronic variant IDE9.

Conclusions: Based on our SNP and haplotype results, we delineate the model that *IDE* promoter and 3' untranslated region/downstream variation may have different effects on *IDE* expression, presumably a relevant endophenotype with disorder-specific effects on AD and T2DM susceptibility.

Background

Insulin degrading enzyme (IDE), also known as insulysin, insulin protease or insulinase, is a 110 kDA zinc-dependent metalloprotease of the M16A subfamily, which is coded by a 122 kb spanning, ubiquitously expressed gene on the distal region of the human chromosome 10 q [1]. Apart from its primary target insulin, IDE competitively hydrolyzes multiple proteins including glucagon, atrial natriuretic factor, transforming growth factor-α, and β-endorphin amylin [2,3] These facts suggest a potentially wide role for IDE in the clearance of hormones and bioactive peptides. In addition to its role in insulin catabolism, IDE has been found to degrade β-amyloid (Aβ) 40 and 42 and the amyloid precursor protein (APP) intracellular domain and to eliminate $A\beta_{42}$ neurotoxic effects [4]. The first evidence that IDE might be involved in $A\beta_{42}$ degradation was found by Kurochkin and Goto in 1994, who demonstrated that purified rat IDE efficiently degrades synthetic $A\beta_{42}$ *in vitro* [3]. Subsequently, it was shown that an IDE-like activity from soluble and synaptic membrane fractions of postmortem human brain both degrade $A\beta_{42}$ peptides [5,6]; moreover, IDE doesn't distinguish between endogenous and synthetic $A\beta_{42}$ as substrate *in vitro* [7]

The fact that extracellular $A\beta_{42}$ deposition is one of the hallmarks of the neurodegenerative disorder Alzheimer's disease (AD) made *IDE* a promising candidate involved in disease susceptibility. Several studies associated linkage-peaks over the *IDE* region with late-onset AD (LOAD) [8], age of onset in familial AD [9] and high plasma $A\beta_{42}$ levels [10]. Furthermore, validated linkage peaks with the metabolic syndrome Type 2

* Correspondence: e_bartl_j@klinik.uni-wuerzburg.de
† Contributed equally
[1]Department of Psychiatry, Psychosomatic and Psychotherapy, University Hospital of Wuerzburg, Fuechsleinstr. 15, D-97080 Wuerzburg, Germany
Full list of author information is available at the end of the article

BioMed Central

© 2011 Bartl et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Diabetes Mellitus (T2DM) were found in the same region [11,12]. Fakhrai-Rad and colleagues narrowed down the wide range of possible candidate genes to *IDE* via transferring the gene from an inbred rat model of T2DM to a normoglycemic rat, which resulted in the recapitulation of several diabetic features, including hyperinsulinemia and postprandial hyperglycemia [13].

In the present study, we undertook an attempt to associate selected variants of the *IDE* gene with AD, $A\beta_{42}$ plasma levels and T2DM in participants of the Vienna Transdanube Aging (VITA) longitudinal cohort study.

Methods

Subjects

Subjects of the present study were from the VITA study which was described previously in greater detail [14,15]. The VITA study investigated the residents of two Viennese districts born between May 1925 and June 1926 (i.e., aged 75 years at inclusion). Data refer to the total cohort at baseline recruitment of 606 individuals who completed physical health check, questionnaires for education, psychosocial activities and neuropsychological examination. The 1st follow-up after 30 months included 468 subjects performing the full health check at the Danube hospital and 30 subjects that were either examined at home or interviewed on the phone. Further 70 subjects refused to attend the follow-up and 38 deceased. At the 2nd follow-up after 60 months 362 subjects attended the full health check at the Danube hospital, whereas 68 subjects were visited at home or only willing to take part in a telephone-interview, thus providing only minimal information. 81 subjects out of the 606 participants deceased between baseline and the 2nd follow-up investigation, 92 subjects refused to take part again and in three cases no contact was possible to establish. A diagnosis of AD was established applying the NINCDS-ADRDA criteria [16]. All participants passed through a consensus conference with regard to the diagnoses of possible or probable AD. The final diagnosis was made by an experienced geronto-psychiatrist. Since the NINCDS-ADRDA diagnosis requires longitudinal information, at baseline all demented subjects were considered as AD positive. In rare cases (n = 16), later (and thus more reliable) examinations revealed "no AD" diagnoses in subjects previously diagnosed as AD positive. For the present analysis, these subjects were treated as AD negative up to the last "no AD" diagnosis. Additional information was obtained in all cases for relevant serum parameters such as cortisol and glucose level and T2DM was diagnosed according to the guide line of the world health organisation. One participant was diagnosed with an untreated schizophrenia; this subject was excluded from AD analyses, however retained in T2DM analyses due to absence of anti-schizophrenic medication, which is known to increase T2DM susceptibility. Additional file 1 - "Demographic information of the VITA study cohort" gives an overview of the subjects examined in this study.

The VITA study was carried out with the permission of the Ethics Committee of the City of Vienna, Austria and each participant gave a written informed consent.

DNA extraction

DNA was prepared from 2 ml EDTA-blood as previously described [15]. Finally, the DNA was aliquoted into cryo-vials (NUNC, Langenselbold, Germany) and stored frozen at -70 C till requirement.

IDE SNP selection and genotyping

The most validated *IDE* SNPS were selected after publication research for association studies in AD and T2DM [17-20]. The *IDE* genotypes were determined using TaqMan assay with the real time PCR reaction using specific primers from TaqMan single nucleotide polymorphism (SNP) Genotyping Assay (Applied Biosystems, Darmstadt, Germany), which uses the 5' nuclease assay for amplifying and detecting specific SNP alleles in purified genomic DNA samples. C_22272896_10 (IDE2, rs4646953), C_27104906_10 (IDE7, rs2251101) and C_12116624_10 (IDE9, rs1887922) assays were used for SNP typing. Analysis of the genotypes was conducted on the iCycler software with allelic discrimination program (Bio-Rad, Munich, Germany).

$A\beta_{1-42}$ plasma level

Plasma levels of amyloid were determined by a double-antibody sandwich enzyme-linked immunosorbent assay method (Innogenetics NV, Ghent, Belgium). The INNOTEST β-amyloid (1-42) allows the specific and reliable measurement of (1-42) amyloid peptides in plasma [21]. The detection range is 5-1000 pg/ml.

Statistical analysis

Prior to association analysis, *IDE* SNPs were tested for Hardy-Weinberg equilibrium (HWE) with a one degree of freedom χ^2-test; no significant departures were detected (all p-values > 0.001). Associations in the different time points were tested with logistic regression when the outcome variable was binary (no/yes), or with linear regression when influence on a continuous outcome was examined. For longitudinal association analysis, generalized estimating equations were used. Genotypic associations were performed using three different models: in the additive model, each individual's risk allele count entered the regression; the dominant model considered the presence of at least one risk allele and in the recessive model, a genotypic risk was only

present if the individual was homozygous for the risk allele. The Akaike Information Criterion (AIC) was used to choose the best model. In single SNP analysis the polymorphism's minor allele was assumed to be the risk allele; all minor allele frequencies were above our inclusion threshold of 5%. In haplotype analysis, each haplotype allele was tested against all other alleles; to account for phase uncertainty, each allele's posterior probability was incorporated into the model. Haplotypes were defined with the expectation-maximization algorithm [22]. Individuals with any missing values in *IDE* genotypes (n = 38 of 606 in the original VITA cohort) were removed from haplotype analyses. Only common haplotypes with a frequency of at least 5% were analyzed. Associations were considered to be significant at α = 0.05; due to the limited sample size of this study, the reported nominal p-values were not adjusted for multiple testing. Power estimates for the determined effect sizes refer to the described analysis settings and result from the comparison of test statistic distributions under the null and alternative hypothesis. All association and power analyses were performed in R version 2.10.0 using the packages geepack, pwr, powerMediation and SimHap (all obtained from http://www.r-project.org).

Results

We examined the associations of *IDE* SNPs (IDE2, rs4646953; IDE7, rs2251101; IDE9, rs1887922) and derived haplotypes (on the coding strand in genomic order 5'-IDE2-IDE9-IDE7-3') with AD and $A\beta_{42}$ plasma levels, as well as with T2DM in unrelated subjects participating in the VITA longitudinal cohort study. Further information can be found in Additional file 2 - "*Insulin degrading enzyme* genotype distributions and tests for Hardy-Weinberg equilibrium" and Additional file 3 -"Power calculations for examined markers and outcomes".

Insulin degrading enzyme polymorphisms and Alzheimer's disease

Genetic associations of *IDE* with AD used dominant models. In order to prove the independence of the observed effects from known risk factors like presence of *APOE* ε4 alleles and female sex, respectively, those covariates were included in multivariate regressions. This revealed a significant association of IDE2 (p = 0.03, see Table 1) in subjects that attended the 60 months follow-up (t = 60). The minor allele (C on the coding strand) was found to have a protective effect (OR = 0.55); longitudinally, the effect size did not undergo significant monotonic changes over time (p = 0.41, see Table 1). Results from haplotype analysis reflect the findings of single marker analysis in that the haplotype that carries the IDE2 risk allele together with the major alleles of IDE9 and IDE7 is also associated with AD at t = 60 (see Table 2): haplotype CAA conveys a significantly (p = 0.02) protective (OR = 0.5) effect. As in single marker analysis, the observed effect size did not undergo significant monotonic changes over time (see Table 2).

Insulin degrading enzyme polymorphisms and amyloid β_{42} plasma level

We then examined the additive influence of *IDE* SNP alleles and haplotypes on the endophenotype $A\beta_{42}$ plasma level. Multivariate regressions included *APOE* ε4

Table 1 Association results of *insulin degrading enzyme* SNPs

		AD		$A\beta_{42}$ plasma level [pg/ml]		T2DM	
Polymorphism (major/minor allele)	focus	OR (95% CI)	p-value	slope (95% CI)	p-value	OR (95% CI)	p-value
IDE2 (T/C)	baseline	-	-	22.36 (8.08 - 36.6)	**0.002**	0.82 (0.23 - 2.86)	0.75
IDE2 (T/C)	1st follow-up	0.66 (0.36 - 1.21)	0.18	21.04 (2.08 - 40)	**0.03**	0.96 (0.3 - 3.05)	0.95
IDE2 (T/C)	2nd follow-up	0.55 (0.32 - 0.95)	**0.03**	35.26 (12.8 -57.8)	**0.002**	1.32 (0.41 - 4.24)	0.64
IDE2 (T/C) × examination	longitudinal	1.13 (0.84 - 1.43)	0.41	8.23 (1.93 -14.5)	**0.01**	1.27 (0.81 - 1.73)	0.31
IDE7 (A/G)	baseline	1.74 (0.63 - 4.78)	0.28	-5.24 (-16.7 - 6.2)	0.37	2.4 (1.25 - 4.63)	**0.009**
IDE7 (A/G)	1st follow-up	1.26 (0.77 - 2.07)	0.35	-5.31 (-21 -10.3)	0.51	2.62 (1.32 - 5.18)	**0.006**
IDE7 (A/G)	2nd follow-up	0.97 (0.62 - 1.52)	0.89	-14.27 (-33.7 - 5.2)	0.15	3.47 (1.72 - 6.97)	**0.0005**
IDE7 (A/G) × examination	longitudinal	0.86 (0.56 - 1.16)	0.33	-3.37 (-8.2 - 1.49)	0.18	1.1 (0.91 - 1.3)	0.32
IDE9 (A/G)	baseline	1.87 (0.71 - 4.92)	0.21	-7.36 (-19.5 - 4.8)	0.24	1.65 (0.72 - 3.79)	0.24
IDE9 (A/G)	1st follow-up	1.02 (0.62 - 1.69)	0.94	-8.39 (-24.9 - 8.1)	0.32	1.17 (0.46 - 3.0)	0.75
IDE9 (A/G)	2nd follow-up	0.99 (0.62 - 1.58)	0.96	-11.1 (-31.8 - 9.7)	0.3	1.75 (0.7 - 4.37)	0.23
IDE9 (A/G)× examination	longitudinal	0.88 (0.57 - 1.19)	0.42	-0.85 (-5.5 - 3.83)	0.72	0.98 (0.78- 1.17)	0.81

Insulin degrading enzyme (IDE) SNPs (IDE2, rs4646953; IDE7, rs2251101; IDE9, rs1887922) were associated with Alzheimer's disease (AD), amyloid β_{42} ($A\beta_{42}$) plasma levels and Type 2 diabetes mellitus (T2DM). Effect sizes and nominal p-values were derived from multivariate regression analysis with sex and presence of *apolipoprotein E (APOE* ε4) alleles as covariates in regressions on AD affection and $A\beta_{42}$ plasma level, respectively. T2DM affection as outcome used sex and BMI as covariates. Longitudinal analysis examined interactions of all covariates with the examination (baseline, 1st and 2nd follow-up). Associations were calculated using dominant models for AD, additive models for $A\beta_{42}$ plasma level and recessive models for T2DM. Nominally significant p-values ($p < 0.05$) are shown in bold.

Table 2 Association results of *insulin degrading enzyme* haplotypes

haplotype				AD		$A\beta_{42}$ plasma level [pg/ml]		T2DM	
5' IDE2	IDE9	'3 IDE7	focus	OR (95% CI)	p-value	Slope (95% CI)	p-value	OR (95% CI)	p-value
T	A	A	baseline	2.01 (0.46 -9.47)	0.34	-5.8 (-16.1 - 4.5)	0.27	1.01 (0.63 -1.61)	0.98
T	A	A	1st follow-up	1.96 (0.98 - 3.9)	0.06	-5.33 (-19.5- 8.86)	0.46	0.83 (0.5 - 1.38)	0.46
T	A	A	2nd follow-up	1.51 (0.85 -2.68)	0.16	-7.37 (-24.7 -9.95)	0.41	0.92 (0.54 -1.59)	0.78
T	A × examination	A	longitudinal	0.94 (0.63 -1.41)	0.77	-0.05 (-0.18- 0.07)	0.4	0.95 (0.84 -1.07)	0.41
T	G	G	baseline	2.23 (0.82 -6.1)	0.12	-6.98 (-20.4- 6.47)	0.31	3.25 (1.31 -8.04)	**0.01**
T	G	G	1st follow-up	1.03 (0.61 -1.74)	0.92	-6.78 (-25.1-11.6)	0.47	2.22 (0.81- 6.05)	0.12
T	G	G	2nd follow-up	0.93 (0.56 -1.53)	0.78	-12.06 (-35.3-1.17)	0.31	3.28 (1.2 - 8.99)	**0.02**
T	G × examination	G	longitudinal	0.82 (0.59 -1.14)	0.25	-0.01 (-0.23- 0.21)	0.92	0.99 (0.79 -1.24)	0.93
T	A	G	baseline	0.91 (0.25 -3.26)	0.89	-0.62 (-17.9-16.7)	0.94	6.0 (1.29 -27.7)	**0.02**
T	A	G	1st follow-up	1.22 (0.66 -2.26)	0.52	-0.75 (-25.13-23.6)	0.95	13.0 (1.47-129.33)	**0.02**
T	A	G	2nd follow-up	1.02 (0.57-1.83)	0.94	-13.76 (-43-15.48)	0.36	8.66 (1.54 -48.77)	**0.01**
T	A × examination	G	longitudinal	0,99 (0.69- 1.43)	0.97	0.24 (-0.45- 0.94)	0.49	1.28 (0.64 - 2.6)	0.49
C	A	A	baseline	-	-	26.36 (10.84-41.88)	**0.001**	0.34 (0.04 -2.63)	0.3
C	A	A	1st follow-up	0.65 (0.34-1.23)	0.18	25.93 (5.21-46.64)	**0.01**	0.59 (0.12 -2.82)	0.51
C	A	A	2nd follow-up	0.5 (0.28- 0.88)	**0.02**	41.5 (17 - 66)	**0.001**	0.87 (0.18- 4.14)	0.86
C	A × examination	A	longitudinal	1.08 (0.81-1.46)	0.59	0.23 (-0.38 -0.86)	0.45	1.26 (0.69 -2.32)	0.45

Insulin degrading enzyme (IDE) haplotypes (IDE2, rs4646953; IDE7, rs2251101; IDE9, rs1887922) with frequencies > 5% were associated with Alzheimer's disease (AD), amyloid β_{42} ($A\beta_{42}$) plasma levels and Type 2 diabetes mellitus (T2DM). Effect sizes and nominal p-values were derived from multivariate regression analysis with sex and presence of *apolipoprotein E (APOE* ε4) alleles as covariates in regressions on AD affection and $A\beta_{42}$ plasma level, respectively. T2DM affection as outcome used sex and BMI as covariates. Longitudinal analysis examined interactions of all covariates with the examination (baseline, 1st and 2nd follow-up). Associations were calculated using dominant models for AD, additive models for $A\beta_{42}$ plasma level and recessive models for T2DM. Nominally significant p-values ($p < 0.05$) are shown in bold.

status and sex as covariates. The analyses concordantly revealed that with each minor C allele of IDE2 the $A\beta_{42}$ plasma level increases between 21.04 and 35.26 pg/ml, depending on time point of analysis (see Table 1 and Figure 1A). This finding was significant in all time points (see Table 1). Longitudinal analysis revealed that the observed effect significantly ($p = 0.01$) increased with each follow-up by 8.23 pg/ml (see Table 1). Of note, an increase in $A\beta_{42}$ plasma level was not exclusive to IDE2 risk allele carriers, but was also observed in individuals homozygous for the major allele (see Table 3 and Figure 1). Since an increased $A\beta_{42}$ plasma level is a known risk factor for AD, we also examined the interaction of the IDE2 genotype with the AD disease status on the $A\beta_{42}$ plasma level. The effect sizes at t = 30 and t = 60 reveal that the IDE2 C allele as well as AD affection both lead to an increased $A\beta_{42}$ plasma level, however that the combined effect is much more pronounced than the sum of both effects (see Table 4 and see Figure 1B). However, the effects for AD and its interaction with the IDE2 genotype are only significant at t = 60, while the effect of IDE2 alone reaches only marginal significance (see Table 4). On multimarker level, we found each CAA haplotype to significantly increase the $A\beta_{42}$ plasma level between 25.93 and 41.5pg/ml, depending on time point of analysis (see table 3B). Longitudinally, we observed no increasing haplotype effect on $A\beta_{42}$ plasma level between examinations (see Table 2).

Insulin degrading enzyme polymorphisms and type 2 diabetes mellitus

Genetic associations of *IDE* with T2DM used recessive models. In order to prove the independence of the observed effects from known risk factors like body mass index (BMI) and female sex, those covariates were included in multivariate regressions. In all three time points, we found the minor G allele of IDE7 to bear a significant ($p<0.01$) genotypic risk to develop T2DM, with an initial (t = 0) OR of 2.4, that continually increased slightly (OR = 1.1) but not significantly ($p = 0.32$) to 3.47 at t = 60 (see Table 1). Haplotype analyses revealed the association of haplotypes TAG and TGG with T2DM, in case of the first in all three time points, in case of the second only at t = 0 and t = 60 (see Table 2). In line with the single marker result for IDE7, both haplotypes convey a genetic risk to become diabetic with significant effect sizes ranging from 3.25 to 3.28 for TGG and from 6 to even 13 for TAG, depending on

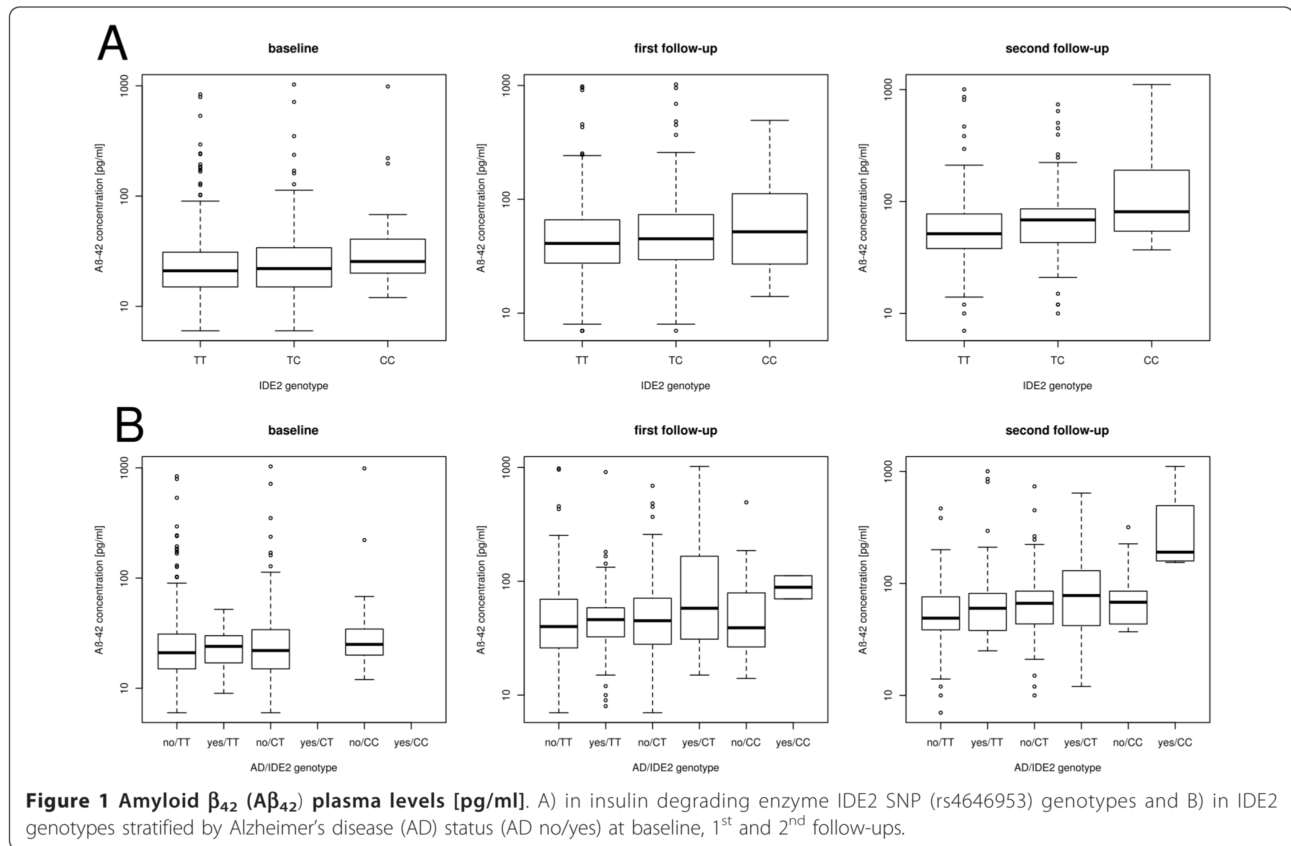


Figure 1 Amyloid β_{42} ($A\beta_{42}$) plasma levels [pg/ml]. A) in insulin degrading enzyme IDE2 SNP (rs4646953) genotypes and B) in IDE2 genotypes stratified by Alzheimer's disease (AD) status (AD no/yes) at baseline, 1st and 2nd follow-ups.

time point of analysis (see Table 2). Longitudinally, the associated haplotypes did not exhibit significant monotonic changes in effect sizes (see Table 2).

Discussion

The identification of a late-onset AD risk locus on chromosome 10q which also affects plasma $A\beta_{42}$ levels, has led to the search for functional candidates in this region [8,10]. Because of its mapping to the AD linkage peak and of the ability of its gene product to degrade $A\beta_{42}$, *IDE* was proposed to harbour a plausible origin for the observed effects. Since *IDE* is also well-known to be associated with increased risk for T2DM [12,23,24], the present study aimed at examining the effects of selected common *IDE* polymorphisms on both disease outcomes

Table 3 Longitudinal analysis of amyloid β_{42} ($A\beta_{42}$) plasma levels in different insulin degrading enzyme 2 (SNP rs4646953) genotypes

IDE2 genotype	$A\beta_{42}$ plasma level [pg/ml]	
	slope (95% CI)	p-value
all genotypes	23.7 (20.34 - 27.07)	< 2 · 10
only T/T	21.33 (17.73 - 24.94)	$< 2 \cdot 10^{-16}$
only T/C	25.84 (16.52 - 35.17)	$5.6 \cdot 10^{-8}$
only C/C	43.28 (27.7 - 58.86)	$5.23 \cdot 10^{-8}$

Effect sizes and nominal p-values were derived from multivariate regression analysis with sex and presence of *apolipoprotein E (APOE* ε4) alleles as covariates. All IDE2 (SNP rs4646953) genotype groups display a highly significant increase in $A\beta_{42}$plasma concentration between each of the three examinations.

Table 4 Interaction analysis of insulin degrading enzyme 2 (SNP rs4646953) genotype

parameter (baseline/ risk)	examination	slope (95% CI)	p-value
IDE2 (T/C)	baseline	20.72 (6.23 - 35.21)	**0.005**
AD (no/yes)		-7.45 (-50.34 - 35.45)	0.73
IDE2 × AD		-	-
IDE2 (T/C)	1st follow-up	13.60 (-6.92 - 34.11)	0.19
AD (no/yes)		7.36 (-23.75 - 38.48)	0.64
IDE2 × AD		33.91 (-22.02 - 89.84)	0.24
IDE2 (T/C)	2nd follow-up	21.41 (-3.43 - 46.25)	0.09
AD (no/yes)		36.27 (3.72 - 68.82)	**0.03**
IDE2 × AD		76.89 (24.01 - 129.77)	**0.005**

Analysis of interaction of insulin degrading enzyme SNP rs4646953 *(IDE2)* genotype and Alzheimer's disease (AD) status on amyloid β_{42} ($A\beta_{42}$) plasma level is shown. Effect sizes and nominal p-values were derived from multivariate regression analysis with sex and presence of *apolipoprotein E (APOE* ε4) alleles as covariates. Nominally significant p-values ($p < 0.05$) are shown in bold.

as well as on the plasma $A\beta_{42}$ level in subjects participating in the longitudinal VITA cohort study. The analyzed polymorphisms should serve as proxies to partly capture the common allelic variation in the up- (IDE2) and downstream (IDE7) region, as well as in the gene body (IDE9) of *IDE*. Our finding that we were not able to detect associations of IDE9 with neither examined trait leads us to the hypothesis that common *IDE* variants influencing the primary structure of the IDE protein (i.e. non-synonymous SNPs and those affecting splicing junctions, respectively) may have no major impact on AD and T2DM susceptibility. However, despite extended linkage disequilibrium (LD) in the *IDE* region (see Additional file 4 - "Linkage disequilibrium in the *insulin degrading enzyme* region"), a single SNP may not fully represent the allelic variation throughout the gene. In fact, 13 tag SNPs of 45 SNPs genotyped in the HapMap CEU panel (release 24) are needed to capture the variation inside IDE with a mean r^2 of 0.99 (data not shown). Therefore the possibility remains that deleterious variants not in LD with IDE9 exist, e.g. rare IDE variants with possibly large effects on our examined traits. While this remains speculative, we found that allelic variation at opposing ends of IDE is associated with different outcomes: the upstream and 5'-untranslated region (UTR) harbours polymorphisms presumably modifying the AD disease risk and the $A\beta_{42}$ plasma level, whereas 3'-UTR and downstream variants may trigger T2DM susceptibility.

There is evidence that the IDE level and therefore $A\beta_{42}$ degrading activity is lower in AD brains than in those of unaffected subjects [6], which can be also an effect of oxidative stress (OS). OS is considered to be a key mechanism in the pathophysiology of AD and is characterized by increased highly reactive oxygen species (ROS) production and decreased antioxidant defence [25,26]. Interestingly the catalytic activity of IDE is reduced through ROS and also the enzymatic activity toward Aβ hydrolysis is decreased [27]. Nonetheless, this has to be further investigated, as it might provide additional link between AD and T2DM. Furthermore, studies on pro-oxidants could be equally important in order to develop new treatment avenues for AD.

A recent study of Zuo and Jia found that variants in the proximal *IDE* promoter increase transcription and thus provide a possible mechanism explaining the protective effect on AD susceptibility found for the examined polymorphisms [28]. Among those, IDE2 overlapped with our study, however with different results: while we found an association with AD in our Vienna-based cohort, IDE2 was not associated in the mentioned Han Chinese sample. This reflects the situation in AlzGene meta-analyses, which list a slightly protective odds ratio (OR) of 0.93 for IDE2 in Central European study populations and an overall OR of 1.0 (i.e. no effect) if Asian studies are included [29]. This makes clear that IDE2 is unlikely to be the causal variant and furthermore raises the possibility that LD between IDE2 and the presumed functional allele varies between Central European and Asian populations. Given this, our present study and that of Zuo and Jia [28] agree that promoter variants leading to increased IDE transcription protect against AD. Correspondingly, one would expect $A\beta_{42}$ plasma levels to be lower in individuals carrying high expression variants, but intriguingly our results show the opposite (see Table 1 and 2). This contradicts the results from a recent study which found high *IDE* expression variants to be correlated with lower Aβ plasma levels, however, $A\beta_{40}$ contributed more to the observation than $A\beta_{42}$ [30]. Plasma $A\beta_{42}$ levels are thus not reliably predicted by *IDE* polymorphisms alone, because also *trans*-acting variants were shown to influence the expression *of IDE* [31]. Furthermore, due to the wide range of IDE substrates, an important determinant for $A\beta_{42}$ degrading activity is the concentration of the primary IDE target (i.e. insulin), illustrated by the finding that even $A\beta_{40}$ clearance is effectively inhibited by insulin [32]. This notion is supported by our observation that a large proportion of the $A\beta_{42}$ plasma level is explained by factors different from the *IDE* promoter genotype (see Table 4).

Hallmarks of T2DM are the presence of insulin resistance and insulin receptor insensitivities. Insulin resistance precedes the onset of T2DM for years [33] and results in compensatory hyperinsulinemia, which is the first step to developing T2DM [34]. Limited capacities to degrade increased plasma insulin levels contribute to development of T2DM. Our association of the downstream variant *IDE7* with T2DM might be an indirect signal that extends from the IDE 3'-UTR over LD. The presumed risk allele may attenuate translation or reduce the stability of the *IDE* mRNA, thus challenging IDE activity and promoting hyperinsulinemia, explaining the increased T2DM susceptibility.

Based on these assumptions, we delineate the model that polymorphisms at opposing ends of the *IDE* gene may lead to expression changes with consequences on susceptibility to different diseases: promoter variation presumably increases *IDE* expression and protects from AD, while 3'-UTR variation is assumed to decrease *IDE* expression and to increase T2DM risk. Correspondingly, haplotypes that carry both associated alleles on a single DNA molecule (5'-CGG-3' and 5'-CAG-3') should reveal a balanced (i.e. no) effect on AD and T2DM. Due to the low frequencies of these haplotypes (0.5% and 0.2%, data not shown) in the VITA study cohort, we were however not able to reliably examine this balancing effect. Despite the plausibility of the model in the context of

the VITA study cohort, validation of the model clearly requires further examinations, including the determination of the IDE activity, which is expected to provide deeper insight into disease causing mechanisms [35].

Conclusions

Based on our SNP and haplotype results, we delineate the model that *IDE* promoter and 3'-UTR/downstream variation may have opposing effects on *IDE* expression, which is assumed to be a relevant endophenotype with disorder-specific effects on AD and T2DM susceptibility. As a starting point for targeted investigations, the present study provides insight how variation in the *IDE* gene contributes to link the pathophysiologically different diseases AD and T2DM.

Additional material

Additional file 1: Demographic information of the VITA study cohort. Abbreviations used: AD = Alzheimer's disease; BMI = body mass index; SD = standard deviation; T2DM = Type 2 diabetes mellitus.

Additional file 2: *Insulin degrading enzyme* genotype distributions and tests for Hardy-Weinberg equilibrium. *Insulin degrading enzyme (IDE)* genotype distributions and tests for Hardy-Weinberg equilibrium (HWE) in analysis subgroups defined by cross-sectional outcomes. No significant departures from HWE were detected (all $p > 0.001$).

Additional file 3: Power calculations for examined markers and outcomes. The presented additional file 3 indicate the power to find nominally significant associations ($p < 0.05$) given analysis settings used for the estimation of effect sizes shown in Tables 1 and 2. Abbreviations used: $A\beta_{42}$ = amyloid beta 1-42 plasma concentration; AD = Alzheimer's disease; IDE2 = rs4646953; IDE7 = rs2251101; IDE9 = rs1887922; SNP = single nucleotide polymorphism.

Additional file 4: Linkage disequilibrium in the *insulin degrading enzyme* region. Linkage disequilibrium (LD) in the *IDE* gene region ± 10 kb is shown based on D' values between single nucleotide polymorphisms (SNPs) genotyped in the HapMap CEU panel (release 24). LD colour scheme corresponds to default settings used in Haploview. Positions of SNPs examined in the present study are indicated in blue. Of note, rs4646953 (IDE2) has not been genotyped in the HapMap project, therefore no LD information is available for this SNP.

Acknowledgements

Funding for this study was provided by the Ludwig Boltzmann Institute (LBI) of Aging Research, Vienna, Austria; the LBI had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. This publication was funded by the German Research Foundation (DFG) and the University of Wuerzburg in the funding programme Open Access Publishing. CJS and SK are supported by the IZKF Wuerzburg. We thank all subjects taking part in the VITA-study and their families. We thank Miryame Hofmann for her excellent technical assistance.

Author details

[1]Department of Psychiatry, Psychosomatic and Psychotherapy, University Hospital of Wuerzburg, Fuechsleinstr. 15, D-97080 Wuerzburg, Germany. [2]IZKF Laboratory for Microarray Applications, University Hospital of Wuerzburg, Versbacher Str. 7, D-97078 Wuerzburg, Germany. [3]Ludwig Boltzmann Society, L. Boltzmann Institute of Aging Research, Vienna, Austria. [4]Department of Psychiatry, Social Medical Center, Danube Hospital, Langobardenstraße 122, A-1220 Vienna, Austria. [5]Hospital of Child and Adolescent Psychiatry, University of Zurich, Neumuensterallee 9, CH-8032 Zurich, Switzerland.

Authors' contributions

Authors EG and PR designed and managed the study, author JB wrote the first draft of the manuscript, author CJS performed the statistical analysis helped to draft the manuscript, authors SK, PF helped to draft the manuscript and discussed the data, authors MH, SJ, IW, MKR, WD, KHT collected the data and samples of the patients and helped to organized the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 21 April 2011 Accepted: 22 November 2011
Published: 22 November 2011

References

1. Espinosa R, Lemons RS, Perlman RK, Kuo WL, Rosner MR, Le Beau MM: **Localization of the gene encoding insulin-degrading enzyme to human chromosome 10, bands q23——q25.** *Cytogenet Cell Genet* 1991, **57(4)**:184-186.
2. Wang DS, Dickson DW, Malter JS: **beta-Amyloid Degradation and Alzheimer's Disease.** *J Biomed Biotechnol* 2006, **2006(3)**:58406.
3. Kurochkin IV, Goto S: **Alzheimer's beta-amyloid peptide specifically interacts with and is degraded by insulin degrading enzyme.** *FEBS Lett* 1994, **345(1)**:33-37.
4. Mukherjee A, Song E, Kihiko-Ehmann M, Goodman JP Jr, Pyrek JS, Estus S, Hersh LB: **Insulysin hydrolyzes amyloid beta peptides to products that are neither neurotoxic nor deposit on amyloid plaques.** *J Neurosci* 2000, **20(23)**:8745-8749.
5. McDermott JR, Gibson AM: **Degradation of Alzheimer's beta-amyloid protein by human and rat brain peptidases: involvement of insulin-degrading enzyme.** *Neurochem Res* 1997, **22(1)**:49-56.
6. Perez A, Morelli L, Cresto JC, Castano EM: **Degradation of soluble amyloid beta-peptides 1-40, 1-42, and the Dutch variant 1-40Q by insulin degrading enzyme from Alzheimer disease and control brains.** *Neurochem Res* 2000, **25(2)**:247-255.
7. Qiu WQ, Walsh DM, Ye Z, Vekrellis K, Zhang J, Podlisny MB, Rosner MR, Safavi A, Hersh LB, Selkoe DJ: **Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation.** *J Biol Chem* 1998, **273(49)**:32730-32738.
8. Myers A, Holmans P, Marshall H, Kwon J, Meyer D, Ramic D, Shears S, Booth J, DeVrieze FW, Crook R, *et al*: **Susceptibility locus for Alzheimer's disease on chromosome 10.** *Science* 2000, **290(5500)**:2304-2305.
9. Li YJ, Scott WK, Hedges DJ, Zhang F, Gaskell PC, Nance MA, Watts RL, Hubble JP, Koller WC, Pahwa R, *et al*: **Age at onset in two common neurodegenerative diseases is genetically controlled.** *Am J Hum Genet* 2002, **70(4)**:985-993.
10. Ertekin-Taner N, Graff-Radford N, Younkin LH, Eckman C, Baker M, Adamson J, Ronald J, Blangero J, Hutton M, Younkin SG: **Linkage of plasma Abeta42 to a quantitative locus on chromosome 10 in late-onset Alzheimer's disease pedigrees.** *Science* 2000, **290(5500)**:2303-2304.
11. Ghosh S, Watanabe RM, Valle TT, Hauser ER, Magnuson VL, Langefeld CD, Ally DS, Mohlke KL, Silander K, Kohtamaki K, *et al*: **The Finland-United States investigation of non-insulin-dependent diabetes mellitus genetics (FUSION) study. I. An autosomal genome scan for genes that predispose to type 2 diabetes.** *Am J Hum Genet* 2000, **67(5)**:1174-1185.
12. Vionnet N, Hani EH, Dupont S, Gallina S, Francke S, Dotte S, De Matos F, Durand E, Lepretre F, Lecoeur C, *et al*: **Genomewide search for type 2 diabetes-susceptibility genes in French whites: evidence for a novel susceptibility locus for early-onset diabetes on chromosome 3q27-qter and independent replication of a type 2-diabetes locus on chromosome 1q21-q24.** *Am J Hum Genet* 2000, **67(6)**:1470-1480.
13. Fakhrai-Rad H, Nikoshkov A, Kamel A, Fernstrom M, Zierath JR, Norgren S, Luthman H, Galli J: **Insulin-degrading enzyme identified as a candidate diabetes susceptibility gene in GK rats.** *Hum Mol Genet* 2000, **9(14)**:2149-2158.
14. Fischer P, Jungwirth S, Krampla W, Weissgram S, Kirchmeyr W, Schreiber W, Huber K, Rainer M, Bauer P, Tragl KH: **Vienna Transdanube Aging "VITA":**

study design, recruitment strategies and level of participation. *J Neural Transm Suppl* 2002, , **62**: 105-116.
15. Grünblatt E, Schlosser R, Fischer P, Fischer MO, Li J, Koutsilieri E, Wichart I, Sterba N, Rujescu D, Moller HJ, *et al*: **Oxidative stress related markers in the "VITA" and the centenarian projects.** *Neurobiol Aging* 2005, **26(4)**:429-438.
16. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM: **Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease.** *Neurology* 1984, **34(7)**:939-944.
17. Gu HF, Efendic S, Nordman S, Ostenson CG, Brismar K, Brookes AJ, Prince JA: **Quantitative trait loci near the insulin-degrading enzyme (IDE) gene contribute to variation in plasma insulin levels.** *Diabetes* 2004, **53(8)**:2137-2142.
18. Feuk L, McCarthy S, Andersson B, Prince JA, Brookes AJ: **Mutation screening of a haplotype block around the insulin degrading enzyme gene and association with Alzheimer's disease.** *Am J Med Genet B Neuropsychiatr Genet* 2005, **136B(1)**:69-71.
19. Bjork BF, Katzov H, Kehoe P, Fratiglioni L, Winblad B, Prince JA, Graff C: **Positive association between risk for late-onset Alzheimer disease and genetic variation in IDE.** *Neurobiol Aging* 2007, **28(9)**:1374-1380.
20. Blomqvist ME, Chalmers K, Andreasen N, Bogdanovic N, Wilcock GK, Cairns NJ, Feuk L, Brookes AJ, Love S, Blennow K, *et al*: **Sequence variants of IDE are associated with the extent of beta-amyloid deposition in the Alzheimer's disease brain.** *Neurobiol Aging* 2005, **26(6)**:795-802.
21. Blasko I, Kemmler G, Jungwirth S, Wichart I, Krampla W, Weissgram S, Jellinger K, Tragl KH, Fischer P: **Plasma Amyloid Beta-42 Independently Predicts Both Late-Onset Depression and Alzheimer Disease.** *Am J Geriatr Psychiatry* .
22. Excoffier L, Slatkin M: **Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population.** *Mol Biol Evol* 1995, **12(5)**:921-927.
23. Karamohamed S, Demissie S, Volcjak J, Liu C, Heard-Costa N, Liu J, Shoemaker CM, Panhuysen CI, Meigs JB, Wilson P, *et al*: **Polymorphisms in the insulin-degrading enzyme gene are associated with type 2 diabetes in men from the NHLBI Framingham Heart Study.** *Diabetes* 2003, **52(6)**:1562-1567.
24. Duggirala R, Blangero J, Almasy L, Dyer TD, Williams KL, Leach RJ, O'Connell P, Stern MP: **Linkage of type 2 diabetes mellitus and of age at onset to a genetic location on chromosome 10q in Mexican Americans.** *Am J Hum Genet* 1999, **64(4)**:1127-1140.
25. Michel TM, Gsell W, Geuder J, Frangou S, Durany N, Kircher T, Sheldrick AJ, Tatschner T, Schneider F, Riederer P, *et al*: **Can enzyme kinetics of prooxidants teach us a lesson about the treatment of Alzheimer's disease: a pilot post-mortem study.** *World J Biol Psychiatry* **11(4)**:677-681.
26. Michel TM, Gsell W, Kasbauer L, Tatschner T, Sheldrick AJ, Neuner I, Schneider F, Grunblatt E, Riederer P: **Increased activity of mitochondrial aldehyde dehydrogenase (ALDH) in the putamen of individuals with Alzheimer's disease: a human postmortem study.** *J Alzheimers Dis* **19(4)**:1295-1301.
27. Ralat LA, Ren M, Schilling AB, Tang WJ: **Protective role of Cys-178 against the inactivation and oligomerization of human insulin-degrading enzyme by oxidation and nitrosylation.** *J Biol Chem* 2009, **284(49)**:34005-34018.
28. Zuo X, Jia J: **Promoter polymorphisms which modulate insulin degrading enzyme expression may increase susceptibility to Alzheimer's disease.** *Brain Res* 2009, **1249**:1-8.
29. Bertram L, McQueen MB, Mullin K, Blacker D, Tanzi RE: **Systematic meta-analyses of Alzheimer disease genetic association studies: the AlzGene database.** *Nat Genet* 2007, **39(1)**:17-23.
30. Carrasquillo MM, Belbin O, Zou F, Allen M, Ertekin-Taner N, Ansari M, Wilcox SL, Kashino MR, Ma L, Younkin LH, *et al*: **Concordant association of insulin degrading enzyme gene (IDE) variants with IDE mRNA, Abeta, and Alzheimer's disease.** *PLoS One* **5(1)**:e8764.
31. Qin W, Jia J: **Down-regulation of insulin-degrading enzyme by presenilin 1 V97L mutant potentially underlies increased levels of amyloid beta 42.** *Eur J Neurosci* 2008, **27(9)**:2425-2432.
32. Shiiki T, Ohtsuki S, Kurihara A, Naganuma H, Nishimura K, Tachikawa M, Hosoya K, Terasaki T: **Brain insulin impairs amyloid-beta(1-40) clearance from the brain.** *J Neurosci* 2004, **24(43)**:9632-9637.
33. Aoki TJ, White RD: **Initiating insulin in patients with type 2 diabetes.** *J Fam Pract* 2007, **56(8 Suppl Hot Topics)**:S12-20.
34. Cefalu WT: **Insulin resistance: cellular and clinical concepts.** *Exp Biol Med (Maywood)* 2001, **226(1)**:13-26.
35. Kim M, Hersh LB, Leissring MA, Ingelsson M, Matsui T, Farris W, Lu A, Hyman BT, Selkoe DJ, Bertram L, *et al*: **Decreased catalytic activity of the insulin-degrading enzyme in chromosome 10-linked Alzheimer disease families.** *J Biol Chem* 2007, **282(11)**:7825-7832.

Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2350/12/151/prepub

doi:10.1186/1471-2350-12-151
Cite this article as: Bartl *et al.*: Disorder-specific effects of polymorphisms at opposing ends of the *Insulin Degrading Enzyme* gene. *BMC Medical Genetics* 2011 **12**:151.

Submit your next manuscript to BioMed Central and take full advantage of:

- **Convenient online submission**
- **Thorough peer review**
- **No space constraints or color figure charges**
- **Immediate publication on acceptance**
- **Inclusion in PubMed, CAS, Scopus and Google Scholar**
- **Research which is freely available for redistribution**

Submit your manuscript at
www.biomedcentral.com/submit

MANUSCRIPT II

The World Journal of Biological Psychiatry, 2012; 00: 1–8

ORIGINAL INVESTIGATION

Alzheimer's disease and type 2 diabetes: Two diseases, one common link?

JASMIN BARTL[1], CAMELIA-MARIA MONORANU[2], ANNE-KRISTIN WAGNER[1], JANN KOLTER[1], PETER RIEDERER[1] & EDNA GRÜNBLATT[1,3]

[1]*Department of Psychiatry, Psychosomatics and Psychotherapy, University Hospital of Würzburg, Würzburg, Germany,* [2]*Department of Neuropathology, Institute of Pathology, University of Würzburg, Würzburg, Germany, and* [3]*Hospital of Child and Adolescent Psychiatry, University of Zurich, Zurich, Switzerland*

Abstract

Objectives. Although Alzheimer's disease (AD) is the most common form of dementia in the elderly, its aetiology remains mostly unknown. A potential pathophysiological mechanism for AD arises from the knowledge that insulin is also synthesized independently in the central nervous system and is involved in the regulation of memory formation. AD may represent a brain-specific form of insulin resistance. *Methods.* We used immunohistochemistry to investigate the numbers of cells expressing insulin receptor β-subunit (IRβ) and phosphorylated PPARγ (PPARγ(p)) in human post-mortem tissue from patients with AD; AD combined with type 2 diabetes mellitus (T2DM); just T2DM , and from aged-matched controls. These numbers were evaluated in frontal cortex and in dorsal/ventral parts of the hippocampus. *Results.* We observed significantly lower numbers of IRβ positive cells in AD cases compared to all other groups in all investigated brain regions. Also significantly more PPARγ(p) positive cells occurred in each patient group compared to control. *Conclusions***.** T2DM and AD may not be directly linked, but may share common histological features including lower numbers of IRβ positive cells and higher numbers of PPARγ(p) positive cells in all investigated brain regions. These observations may at least partially explain the increased frequency of AD in elderly diabetic patients.

Key words: *Alzheimer´s disease, type 2 diabetes mellitus, insulin receptor* β *subunit, phosphorylated PPAR*γ*, immunohistochemistry staining*

Introduction

The association between type 2 diabetes mellitus (T2DM) and increased risk of dementia in the elderly is well documented (Biessels and Kappelle 2005; de la Monte and Wands 2005). Multiple possible mechanisms for this association have been proposed, including the direct effects of hyperglycaemia, insulin resistance, and insulin-induced amyloid-peptide amyloidosis in the brain (Luchsinger and Gustafson 2009). Alzheimer's disease (AD) is the most common form of dementia in the elderly; despite decades of intense research, the aetiology of AD remains mostly unknown. Since insulin is also synthesised in the central nervous system and is involved in the regulation of several cell processes including memory formation, it may contribute to an underlying pathophysiological mechanism for AD.

An early correlation between insulin receptor β-subunit (IRß) and AD arose in the mid-1980s when it was reported that the hippocampus and parts of the cerebral cortex, regions important to learning and memory, contained high densities of IRβ (Gammeltoft et al. 1985; Baskin et al. 1987). Insulin has dramatic effects on human cognition (Strachan et al. 1997), and the actions of insulin and insulin-sensitising peroxisome proliferator-activated receptor-γ (PPARγ) agonists have been explored in AD patients (Watson and Craft 2003). The risk of AD and memory impairments is increased by hyperinsulinaemia and insulin resistance, characteristics of T2DM (de la Monte 2009; Carlsson 2010), which is associated with an increased risk of AD (Sims-Robinson et al. 2010). These observations and other findings have led to the hypothesis that the cognitive deficits observed in AD may arise, in part, from insulin insensitivity in the brain.

Glucose uptake and metabolism have been shown to be impaired in brain regions involved in memory

Correspondence: PD Dr. Edna Grünblatt, Hospital of Child and Adolescent Psychiatry University of Zurich, Head of the Neurobiochemistry Laboratory, Thurgauerstrasse 39, CH-8050 Zurich, Switzerland. Tel: + 41 (0) 44 578 60 71 or (0) 43 556 40 38. E-mail: Edna.Gruenblatt@kjpdzh.ch

(Received 5 July 2011; accepted 12 December 2011)

ISSN 1562-2975 print/ISSN 1814-1412 online © 2012 Informa Healthcare
DOI: 10.3109/15622975.2011.650204

and cognition in AD patients (Arnaiz et al. 2001; Watson and Craft 2004). For example, insulin can act both *in vitro* and in AD patients to regulate amyloid β (Aβ) levels, and insulin may facilitate Aβ release and interfere with insulin degrading enzyme (IDE)-mediated degradation of Aβ (Qiu and Folstein 2006). In addition, vascular dysfunction associated with insulin resistance may also increase susceptibility to AD (Sa-Roriz et al. 2009; Umegaki 2009). The principal action of PPARγ agonists in the periphery of the body is to enhance insulin sensitivity and lower serum glucose levels, actions that underlie the efficacy of these drugs (Berger and Wagner 2002) and provide a rationale for the use of PPARγ agonists in treating AD.

In this study, we focused on IRβ and phosphorylated PPARγ producing cells in human post-mortem brain tissue. Using immunohistochemical staining against IRβ and PPARγ(p) in brain tissue originating from patients with AD, with AD combined with T2DM, with T2DM, and from aged-matched controls, we evaluated the alterations of these proteins in the frontal cortex and in the dorsal and ventral parts of the hippocampus.

Materials and methods

Sample preparation

The brain samples used in this study were supplied by Brain Net Europe. The entire procedure was performed in accordance with the Helsinki Declaration in its latest version and with the Convention of the Council of Europe on Human Rights and Biomedicine. Clinical diagnosis of AD was based on NINCDS-ADRDA criteria and confirmed by neuropathological findings (Braak and Braak 1991).

Histological samples were obtained from the dorsal and ventral hippocampus and from the prefrontal cortex of post-mortem brains from four patient groups (Supplementary Table 1 available online at http://www.informahealthcare.com/doi/abs/10.3109/15622975.2011.650204): age-matched control patients without dementia, patients with T2DM, AD patients, and patients with AD plus T2DM. In preparation for routine neuropathological examination, the brain was divided midsagittally, and one hemisphere was immersed in 4.5% *p*-formaldehyde (Fischer GmbH, Saarbruecken, Germany) for 3–4 weeks.

Immunohistochemistry

Paraffin sections (8 μm) of post-mortem brain tissue from the four patient groups were deparaffined with an alcohol dilution series. The slides were then boiled in 10 mM citrate buffer cointaing 10.51 g citrate monohydrate and 2 g sodium hydroxide pellets in 5 l double distillate water (pH 6) for at least 10 min for antigen retrieval. The sections were washed three times in Tris-buffered saline, and non-specific binding was blocked with blocking solution containing 10% normal goat serum, 2% bovine serum albumin, and 0.01% Triton X-100 in Tris-buffered saline for 1 h before incubation with the various primary antibodies diluted in blocking solution. The primary antibodies targeted the following proteins: IRβ (Santa Cruz, Heidelberg, Germany), diluted 1:200; neuronal specific enolase (NSE; Abcam, Cambridge, UK), diluted 1:300; and PPARγ(p) (Abcam, Cambridge, UK), diluted 1:200 and all antibodies were incubated overnight at 4°C. The primary antibodies were visualised with antibodies conjugated to Alexa® Fluor-488 (green) and Fluor-555 (red; Invitrogen, Darmstadt, Germany). All secondary antibodies were incubated for 2 h at room temperature in the dark. Finally, the sections were mounted on glass slides and cover slipped under Vectashield (Vector Labs, Eiching, Germany) for fluorescence microscopy.

Automated cell counting

Five images of each brain region from each patient were recorded and analysed with Cell^P (version 2.0; Olympus, Hamburg, Germany). We used Imaging C within Cell^P to use a macro recorder, to define special regions of interest for each investigated protein, and to perform automated cell counting. The total cell number for each image was determined, and we separately obtained counts for the special regions of interest for each image. Automated cell counting helped us maintain the standard error of measurement at the same level for image analyses.

Statistical analysis

Immunohistochemistry images were analysed for the numbers of cells positive for NSE, IRβ, and PPARγ(p) staining in comparison to the total number of cells via analysis of variance (ANOVA) and a post-hoc Scheffé test with a significance level of $P < 0.05$. Reported values were calculated in relative to control levels (100%). The statistical program Stat View 5.0 (SAS Institute Inc. Cary, NC, USA) was used for all analyses.

Results

Figure 1 represents one example of histochemical staining for NSE in the prefrontal cortex of a control case. Fluorescent pictures were always recorded in gray values for counting. Each protein had its own

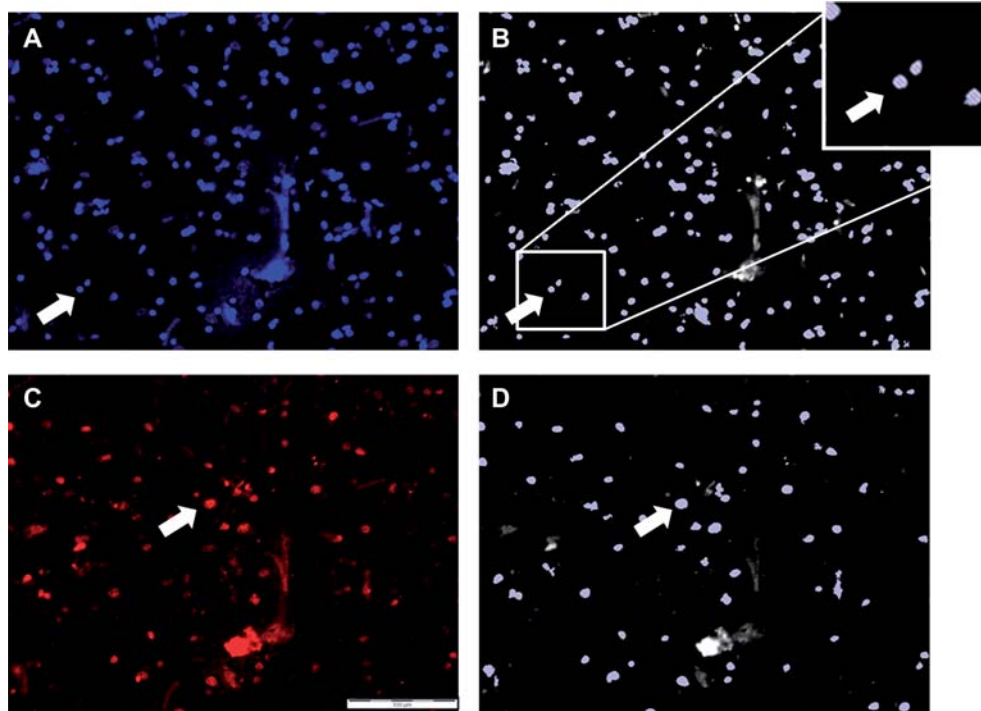


Figure 1. Immunohistochemical staining of neuronal specific enolase (NSE) in the prefrontal cortex of a control case. Five fluorescent images of each brain region were recorded for each case. The dashed area was included in the automated cell counting. (A) Cell nucleus stained in blue; (B) cell nucleus in gray values; (C) NSE staining in red; (D) NSE staining in gray values. The arrows point to the same structure in the fluorescent and gray images. Scale bar size is 200 μm.

specific counting parameters, which were computationally defined for automated counting.

In order to ascertain that neuronal cell loss was not the predominant factor in our analysis of patient groups and control subjects, we stained all brain section slides (prefrontal cortex, dorsal and ventral hippocampus) with NSE antibody in all patient groups. In the prefrontal cortex we observed a minor but non-significant difference in the number of enumerated neurons in all three patients groups compared to the age-matched controls (Figure 2). AD patients had 17% fewer NSE positive cells, AD plus T2DM patients had 10% fewer NSE-positive cells, and T2DM patients had 15% fewer NSE-positive cells than the age-matched control subjects (Figure 2). Compared to the control group, the dorsal and ventral hippocampus sections contained higher numbers of NSE positive cells in T2DM patients (24–28% more) and in patients with AD plus T2DM (14% more), but these differences were not statistically significant (Figure 2). We used these brain regions for subsequent investigations because they were relatively unaffected by neuronal cell lost in all groups, enabling identification and confirmation of specific protein alterations.

Cognitive function is an important parameter in the evaluation and determination of AD progression; the prefrontal cortex and hippocampus are critical regions in AD pathology, and for this reason we focused our IRβ quantification on these brain areas. Patients with AD had 50–60% fewer IRβ positive cells in the prefrontal cortex ($P < 0.0001$) compared to the control group as well as compared to the patients group with only T2DM and to the AD + T2DM combined group (Figure 3). A 50% less IRβ in the dorsal ($P = 0.003$) and 60% less IRβ in the ventral hippocampus was found in comparison to the healthy control group ($P < 0.0001$) (Figure 3). While in the dorsal hippocampus we could find only a tendency for less IRβ positive cells in the AD group compared to the combined group ($T = 0.063$). On the other hand, in ventral hippocampus a nominal significance reduction by 20% of IRβ positive cells was detected in the AD group in comparison to the combined group ($P = 0.0051$) (Figure 3). Patients with T2DM alone possessed 32–35% fewer IRβ positive cells in the dorsal hippocampus ($P = 0.031$) and ventral ($P = 0.0103$) compared to control subjects (Figure 3); these values in the same brain regions were 26% ($P = 0.0157$) and 27% ($P = 0.0154$) for patients with AD plus T2DM (Figure 3).

Gender-based differences in the number of IRβ positive cells were observed in AD patients (Supplementary Table 2 available online at http://www.informahealthcare.com/doi/abs/10.3109/15622975.2011.650204). In all investigated brain regions,

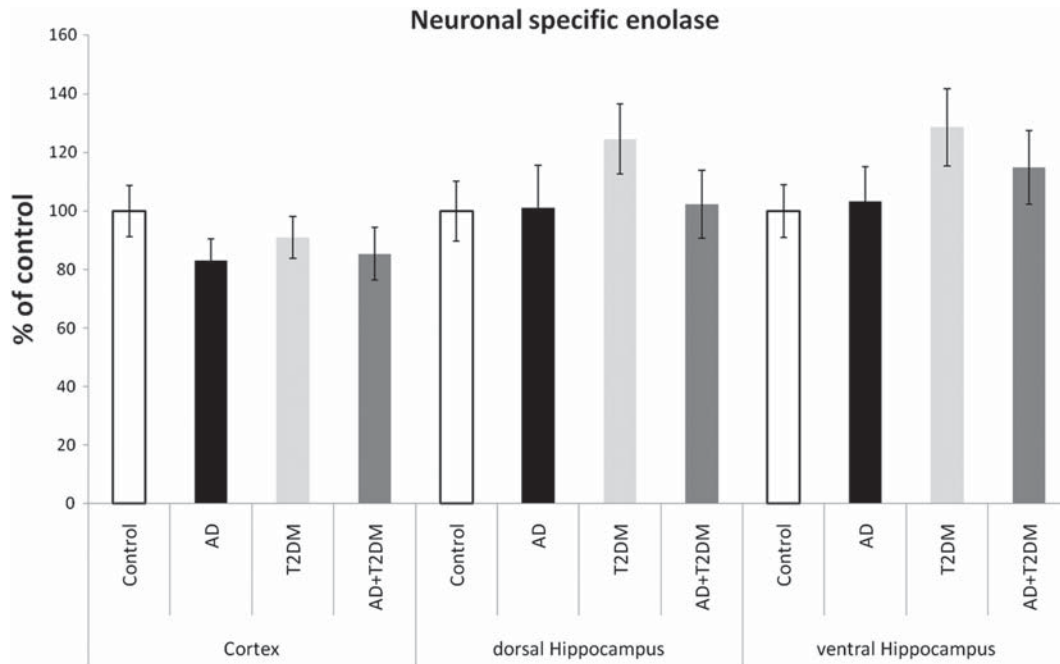


Figure 2. Neuronal specific enolase (NSE) positive cells in post-mortem human brain samples. Using immunohistochemical staining against NSE in brain tissue originating from patients with Alzheimer's disease (AD), with AD combined with type 2 diabetes mellitus (T2DM), patients with T2DM alone, and age-matched controls, alterations in NSE positive cells in the prefrontal cortex and in the dorsal and ventral parts of the hippocampus were evaluated. No statistical differences were found between the three patient groups and the control group in any of the investigated brain regions.

female AD patients had significantly fewer IRβ-positive cells than male AD patients. In the prefrontal cortex females AD patients harboured 15% fewer IRß positive cells, in the dorsal hippocampus they had 16% fewer positive cells, and in the ventral hippocampus we observed 11% fewer positive cells. There was no significant relationship between age and IRβ level in any of the investigated brain regions

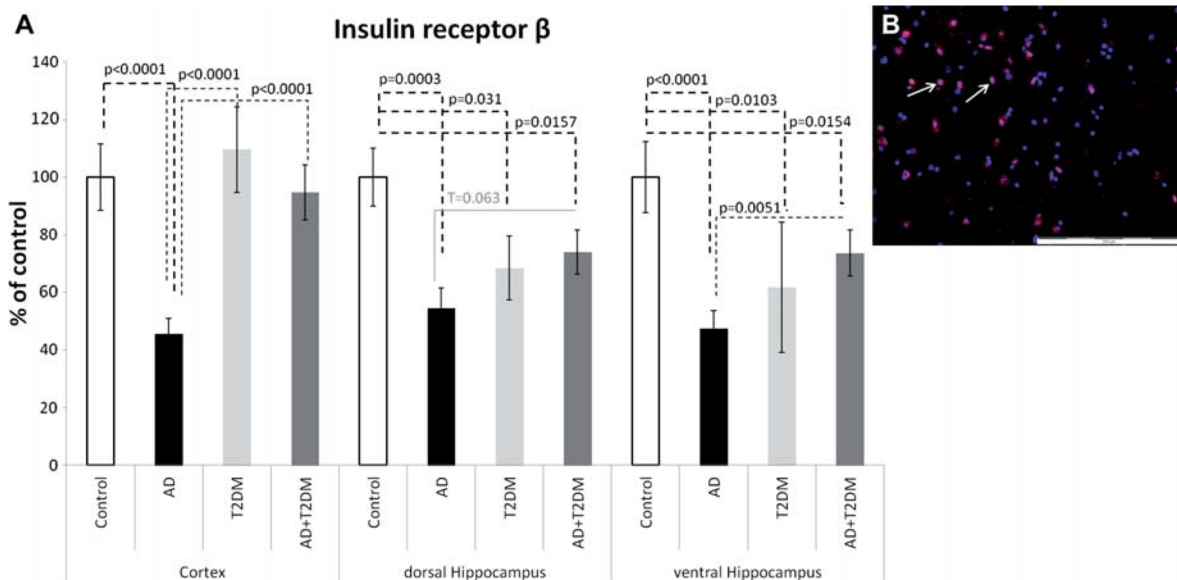


Figure 3. Insulin receptor (IR) β-positive cells in post-mortem human brain samples. Using immunohistochemical staining against IRβ in brain tissue originating from patients with Alzheimer's disease (AD), with AD combined with type 2 diabetes mellitus (T2DM), patients with T2DM alone, and age-matched controls, alterations in NSE positive cells in the prefrontal cortex and in the dorsal and ventral parts of the hippocampus were evaluated. Statistical analysis of IRβ positive cells was performed using ANOVA, post hoc Scheffé; – – – = $P < 0.05$; T= nominal significance; $n = 10$ cases/group. (B) Immunohistochemical staining of IRβ in hippocampus of a control case. Cell nucleus is stained in blue; IRβ is stained in red. The arrows point to the right structure. Scale bar size is 200 μm.

(Supplementary Table 3 available online at http://www.informahealthcare.com/doi/abs/10.3109/15622975.2011.650204).

The most significant results involved the analysis of PPARγ(p) positive cells. We detected more than 60% more PPARγ(p) positive cells in all patients group ($P < 0.001$) compared with the control group (Figure 4). There were no gender-specific differences in the number of PPARγ(p) positive cells in the cortex, but analysis of the dorsal and ventral hippocampus revealed significant differences (Supplementary Table 2 at http://www.informahealthcare.com/doi/abs/10.3109/15622975.2011.650204.). Female patients with T2DM had 10% fewer PPARγ(p) positive cells than male patients; in contrast, female AD patients possessed 13–18% more PPARγ(p) positive cells in these brain regions.

Discussion

Accumulating evidence supports the hypothesis that AD and T2DM share a common link (de la Monte 2009; Sa-Roriz et al. 2009; Sims-Robinson, Kim et al. 2010); while aging is clearly the strongest risk factor for AD, emerging data suggest that T2DM and dyslipidaemic states can contribute substantially to AD pathogenesis either directly or as cofactors (Qiu et al. 2007). Those observations are not without controversy, and a longitudinal survey revealed that although marginal diabetics had a significantly increased risk for future development of full diabetes, dementia, or AD, the risk effects were independent rather than linked (Xu et al. 2007).

In the present work we observed several common histological features in patients with diabetes and AD. In groups of patients with AD, with T2DM plus AD, and with T2DM alone, the number of IRβ positive cells in the hippocampus was significantly decreased compared to the control group (Figure 3). After evaluation of the total neuronal cell numbers in samples from all four groups, we were able to confirm that the number of IRβ positive cells was affected by AD and T2DM.

Why should AD or T2DM patients have fewer IRβ positive brain cells compared to age-adapted controls? AD is characterized both by low insulin levels and by insulin resistance within the central nervous system that causes a reduction in brain insulin. Several mechanisms may explain why insulin mediates memory facilitation. As noted, insulin receptors are found in brain areas responsible for cognition. Insulin activates signalling pathways associated with learning and long-term memory and helps to regulate processes such as neuronal survival, energy metabolism, and plasticity, processes that are required for learning and memory (Figure 5) (de la Monte and Tong 2009). Examination of post-mortem cases of late-stage AD demonstrated that advanced AD was associated with strikingly reduced levels of

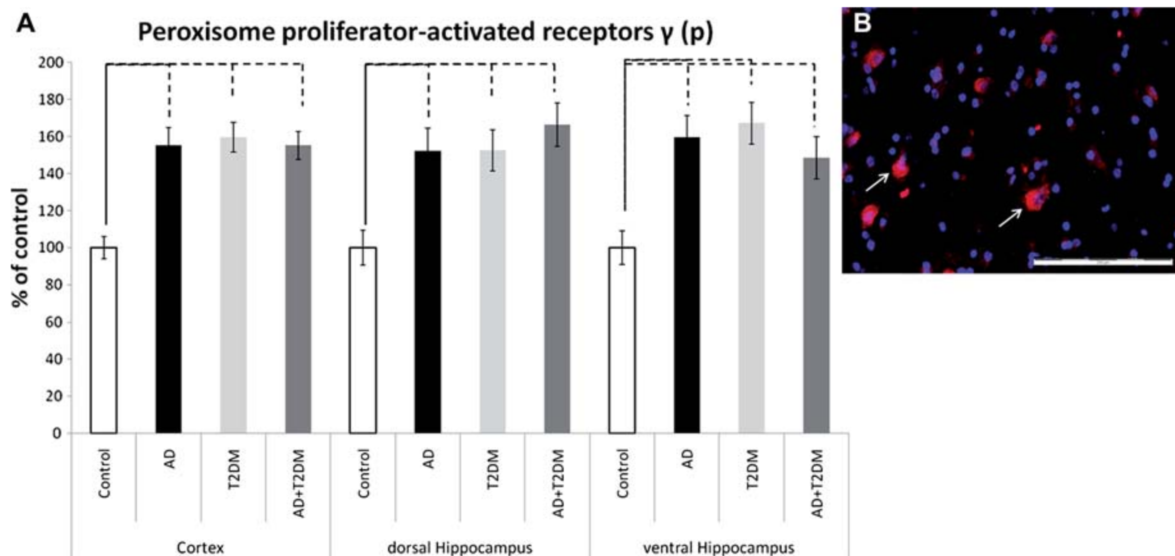


Figure 4. A. Phosphorylated peroxisome proliferator-activated receptor-γ (PPARγ(p))-positive cells in post-mortem human brain samples. Using immunohistochemical staining against PPARγ(p) in brain tissue originating from patients with Alzheimer's disease (AD), with AD combined with type 2 diabetes mellitus (T2DM), patients with T2DM alone, and age-matched controls, alterations in PPARγ(p) positive cells in the prefrontal cortex and in the dorsal and ventral parts of the hippocampus were evaluated. Statistical analysis of PPARγ(p) positive cells was performed using ANOVA; post hoc Scheffé - - - = $P < 0.0001$; $n = 10$ cases/group. (B) Immunohistochemical staining of PPARγ(p) in hippocampus of a control case. Cell nucleus is stained in blue; PPARγ(p) is stained in red. The arrows point to the right structure. Scale bar size is 200 μm.

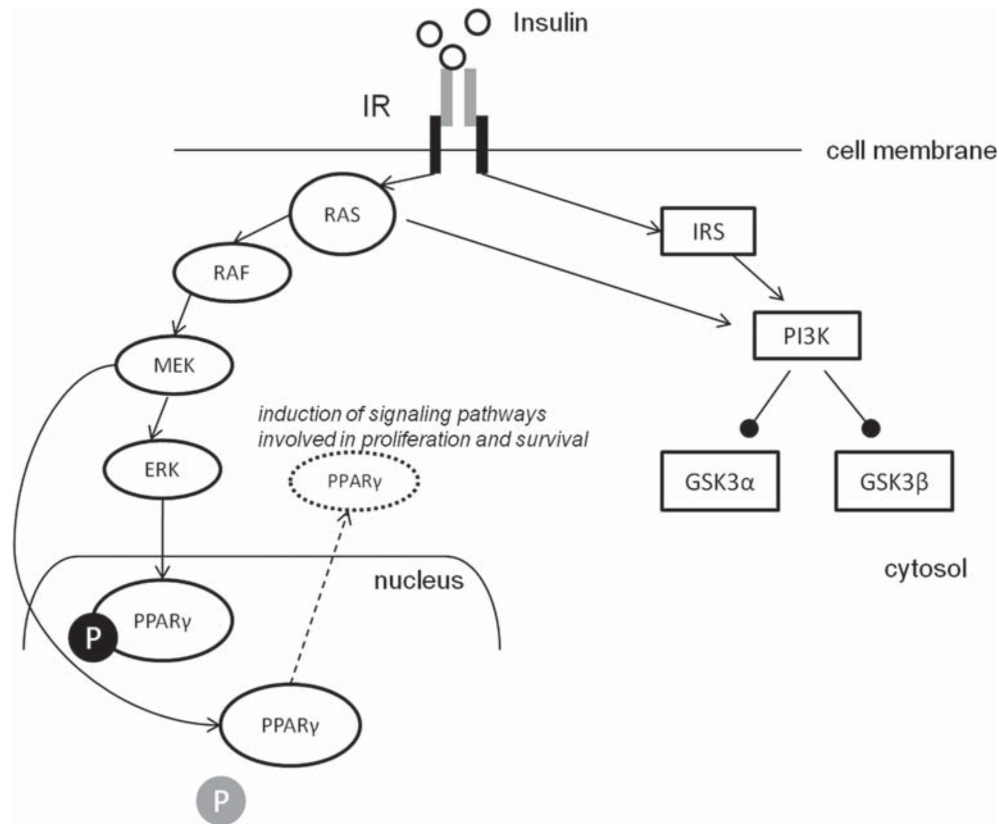


Figure 5. Schematic representation of components of the system encompassing the insulin receptor (IR) and peroxisome proliferator-activated receptor (PPAR). Interactions between PPAR, MAPK/PI3K, and the IR pathways occur at various levels and are indicated as arrows (activation) or dots (inhibition). P = phosphorylated. → = activation; —• = inhibition; ⇢ = transport from nucleus into cytoplasma

insulin, IGF-1 polypeptide, and related receptor genes in the whole brain (Steen et al. 2005).

In the current study we examined whether patients with T2DM shared common histological features with AD, such as fewer IRβ positive brain cells, and whether this patient group harboured significantly fewer IRβ positive cells in the hippocampus but not in the prefrontal cortex. We were unable to confirm our expectation that patients with AD plus T2DM had significantly fewer IRβ positive cells than the single-disease groups, but these patients had significantly fewer IRβ positive cells in the hippocampus. Thus, it seems that IRβ loss in the brain is a restricted phenomenon, and cannot decrease past a certain level. Interestingly, only the hippocampus (not the prefrontal cortex) was affected in patients with T2DM plus AD. Conditions caused by insulin abnormalities, such as T2DM, are associated with an increased risk of age-related cognitive decline (Luchsinger et al. 2001; Peila et al. 2002), possibly due to the lower numbers of IRβ positive cells in the hippocampus of T2DM patients observed in this study. We also calculated the ratio of IRβ positive cells against number of all detected neurons. This analysis indicates that more IRβ positive cells are detected than neuron (Supplementary Table 4 available online at http://www.informahealthcare.com/doi/abs/10.3109/15622975.2011.650204) in all brain regions of each group. This of course, might be due to IR expression in glia cells in addition to the expression in the neuronal cells. It is known that glia cells can express insulin receptors as well (Verdier and Penke 2004). In our study we focused on the changes in neuronal levels of IRβ and did not measure glial alterations. Still since it is documented in the literature that in AD there is an increase in glial cells (Vehmas et al. 2003; Ryu et al. 2009; Venneti et al. 2009), it is rather less likely that our findings might occur due to changes in glial cells. However we cannot exclude the possibility whether there can be a "selective" neuronal cell lost of only IRβ positive neurons in AD. Since this is a very interesting question, we will investigate this topic further on.

Additionally, all patient groups possessed significantly more PPARγ(p) positive cells than the control group in all investigated brain regions. We were interested in PPARγ(p) because 6 months of treatment with rosiglitazone, a typical T2DM treatment and a PPARγ agonist, preserved cognitive function

for patients with AD and amnestic mild cognitive impairment compared with a placebo-treated group (Watson et al. 2005). A number of studies have examined potential mechanisms by which PPARγ agonists may ameliorate AD pathogenesis and progression (Landreth 2007; Neumann et al. 2008); PPARγ agonists improve insulin sensitivity by decreasing the level of circulating insulin, increasing insulin-mediated glucose uptake, and enhancing insulin action in the brain, resulting in cognitive improvement (Sato et al. 2011). The phosphorylated isoform of PPARγ is the inactive variant of this protein (Figure 5) (Adams et al. 1997), and therefore it was not surprising to observe higher numbers of PPARγ(p) positive cells in the brains of all patient groups compared to the control group. However, we were unable to detect between-patient group differences, suggesting that PPARγ(p) is equally affected in AD and T2DM and may be why the PPARγ agonist had positive effects in AD patients as well as in T2DM patients. In our particular study, we focused on the phosphorylated form of PPARγ , which seems to play the important role in the pathophysiology of T2DM and maybe also in AD, but further investigations about the ration between phophorylated and non-phosphorylated PPARγ will be necessary and will be done in our laboratory.

In conclusion, T2DM and AD may not be directly linked, but may share common histological features including lower numbers of IRβ positive cells in the hippocampus and higher numbers of PPARγ(p) positive cells in the prefrontal cortex and the hippocampus. These observations may at least partially explain the increased frequency of AD in elderly diabetic patients.

Acknowledgements

The authors express their thanks for post-mortem brain tissue samples provided by BrainNet Europe II. We also add a special thank you to Mrs Michaela Hartmann and Mrs Hannelore Schraut for their excellent technical work and help.

Statement of Interest

None to declare.

References

Adams M, Reginato MJ, Shao D, Lazar MA, Chatterjee VK. 1997. Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. J Biol Chem 272:5128–5132.

Arnaiz E, Jelic V, Almkvist O, Wahlund LO, Winblad B, Valind S, et al. 2001. Impaired cerebral glucose metabolism and cognitive functioning predict deterioration in mild cognitive impairment. Neuroreport 12:851-855.

Baskin DG, Figlewicz DP, Woods SC, Porte D, Jr., Dorsa DM. 1987. Insulin in the brain. Annu Rev Physiol 49:335–347.

Berger J, Wagner JA. 2002. Physiological and therapeutic roles of peroxisome proliferator-activated receptors. Diabetes Technol Ther 4(2):163–174.

Biessels GJ, Kappelle LJ. 2005. Increased risk of Alzheimer's disease in Type II diabetes: insulin resistance of the brain or insulin-induced amyloid pathology? Biochem Soc Trans 33 (Pt 5):1041–1044.

Braak H, Braak E. 1991. Demonstration of amyloid deposits and neurofibrillary changes in whole brain sections. Brain Pathol 1(3):213–216.

Carlsson CM. 2010. Type 2 diabetes mellitus, dyslipidemia, and Alzheimer's disease. J Alzheimers Dis 20(3):711–722.

de la Monte SM. 2009. Insulin resistance and Alzheimer's disease. BMB Rep 42(8):475–481.

de la Monte SM, Tong M. 2009. Mechanisms of nitrosamine-mediated neurodegeneration: potential relevance to sporadic Alzheimer's disease. J Alzheimers Dis 17(4):817–825.

de la Monte SM, Wands JR. 2005. Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. J Alzheimers Dis 7(1):45–61.

Gammeltoft S, Fehlmann M, Van Obberghen E. 1985. Insulin receptors in the mammalian central nervous system: binding characteristics and subunit structure. Biochimie 67: 1147–1153.

JSR-F, Sa-Roriz TM, Rosset I, Camozzato AL, Santos AC, Chaves ML, et al. 2009. (Pre)diabetes, brain aging, and cognition. Biochim Biophys Acta 1792:432–443.

Landreth G. 2007. Therapeutic use of agonists of the nuclear receptor PPARgamma in Alzheimer's disease. Curr Alzheimer Res 4(2):159–164.

Luchsinger JA, Gustafson DR. 2009. Adiposity, type 2 diabetes, and Alzheimer's disease. J Alzheimers Dis 16(4):693–704.

Luchsinger JA, Tang MX, Stern Y, Shea S, Mayeux R. 2001. Diabetes mellitus and risk of Alzheimer's disease and dementia with stroke in a multiethnic cohort. Am J Epidemiol 154: 635–641.

Neumann KF, Rojo L, Navarrete LP, Farias G, Reyes P, Maccioni RB. 2008. Insulin resistance and Alzheimer's disease: molecular links & clinical implications. Curr Alzheimer Res 5:438–447.

Peila R, Rodriguez BL, Launer LJ. 2002. Type 2 diabetes, APOE gene, and the risk for dementia and related pathologies: The Honolulu-Asia Aging Study. Diabetes 51: 1256–1262.

Qiu C, De Ronchi D, Fratiglioni L. 2007. The epidemiology of the dementias: an update. Curr Opin Psychiatry 20: 380–385.

Qiu WQ, Folstein MF. 2006. Insulin, insulin-degrading enzyme and amyloid-beta peptide in Alzheimer's disease: review and hypothesis. Neurobiol Aging 27(2):190–198.

Ryu JK, Cho T, Choi HB, Wang YT, McLarnon JG. 2009. Microglial VEGF receptor response is an integral chemotactic component in Alzheimer's disease pathology. J Neurosci 29: 3–13.

Sato T, Hanyu H, Hirao K, Kanetaka H, Sakurai H, Iwamoto T. 2009. Efficacy of PPAR-gamma agonist pioglitazone in mild Alzheimer disease. Neurobiol Aging 32(9):1626–33.

Sims-Robinson C, Kim B, Rosko A, Feldman EL. 2010. How does diabetes accelerate Alzheimer disease pathology? Nat Rev Neurol 6:551–559.

Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, et al. 2005. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? J Alzheimers Dis 7:63–80.

Strachan MW, Deary IJ, Ewing FM, Frier BM. 1997. Is type II diabetes associated with an increased risk of cognitive dysfunction? A critical review of published studies. Diabetes Care 20:438–445.

Umegaki H. 2009. Pathophysiology of cognitive dysfunction in older people with type 2 diabetes: vascular changes or neurodegeneration? Age Ageing 39(1):8–10.

Vehmas AK, Kawas CH, Stewart WF, Troncoso JC. 2003. Immune reactive cells in senile plaques and cognitive decline in Alzheimer's disease. Neurobiol Aging 24:321–331.

Venneti S, Lopresti BJ, Wang G, Hamilton RL, Mathis CA, Klunk WE, et al. 2009. PK11195 labels activated microglia in Alzheimer's disease and in vivo in a mouse model using PET. Neurobiol Aging 30:1217–1226.

Verdier Y, Penke B. 2004. Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. Curr Protein Pept Sci 5(1):19–31.

Watson GS, Cholerton BA, Reger MA, Baker LD, Plymate SR, Asthana S, et al. 2005. Preserved cognition in patients with early Alzheimer disease and amnestic mild cognitive impairment during treatment with rosiglitazone: a preliminary study. Am J Geriatr Psychiatry 13:950–958.

Watson GS, Craft S. 2003. The role of insulin resistance in the pathogenesis of Alzheimer's disease: implications for treatment. CNS Drugs 17(1):27–45.

Watson GS, Craft S. 2004. Modulation of memory by insulin and glucose: neuropsychological observations in Alzheimer's disease. Eur J Pharmacol 490(1–3):97–113.

Xu W, Qiu C, Winblad B, Fratiglioni L. 2007. The effect of borderline diabetes on the risk of dementia and Alzheimer's disease. Diabetes 56:211–216.

Supplementary material available online

Supplementary Tables 1–4 to be found available online at http://www.informahealthcare.com/doi/abs/10.3109/15622975.2011.650204.

Supplementary material for Bartl J., Monoranu C-M., Wagner A-K., Kolter J., Riederer P. & Grünblatt E. Alzheimer's disease and type 2 diabetes: Two diseases, one common link? Biol Psychiatry, 2012; DOI: 10.3109/15622975.2011.650204.

Supplementary Table 1: Demographic data of postmortem brains; AD = Alzheimer's disease; T2DM = type 2 diabetes mellitus; m = male; f = female; **SEM = standard error of mean**

	Control	AD	AD + T2DM	T2DM
Age, years **[mean (+/−SEM)]**	69 (+/−9)	75 (+/−7)	75 (+/−6)	71 (+/−6)
Gender (m/w)	4/5	5/5	6/4	4/6
Braak	0-I	IV-VI	III-V	0-I

Supplementary Table 2: Gender differences in insulin receptor (IR) ß positive cells and peroxisome proliferator−activated receptor−γ (PPARγ) phosphorylated (p) in post−mortem human brain.

Region	Diagnose	gender	PPARγ (p) MW(%)	IRβ MW(%)
Cortex	Control	m	49.89 (+/−3.31)	50.34 (+/−6.75)
		f	49.07 (+/−2.61)	47.26 (+/−2.94)
	AD	m	72.73 (+/−6.29)	**33.15 (+/−2.03)**
		f	78.8 (+/−4.43)	**18.76 (+/−1.82) [p = 0.0003]**
	T2DM	m	81.91 (+/−3.34)	52.37 (+/−8.36)
		f	74.56(+/−1.95)	56.75 (+/−4.15)
	AD + T2DM	m	77.6−(+/−4.36)	51.01 (+/−3.75)
		f	76.36 (+/−1.94)	41.91 (+/−3.78)
dorsal Hippocampus	Control	m	**58.37 (+/−2.43)**	46.65 (+/−5.73)
		f	**39.48 (+/−4.96) [p = 0.0019]**	46.11 (+/−3.71)
	AD	m	65.530(+/−5.97)	**36.94 (+/−7.06)**
		f	78.83 (+/−4.10)	**20.63 (+/−1.55) [p = 0.0038]**
	T2DM	m	**80.56 (+/−2.63)**	29.57 (+/−4.72)
		f	**69.24 (+/−3.03) [p = 0.0127]**	42.70 (+/−15)
	AD + T2DM	m	83.73 (+/−3.31)	**29.04 (+/−2.63)**
		f	79.5−(+/−2.52)	**41.95 (+/−3.41) [p = 0.0083]**
ventral Hippocampus	Control	m	53.31 (+/−3.45)	33.40 (+/−5.29)
		f	43.40 (+/−4.80)	47.64 (+/−4.51)
	AD	m	**63.0−(+/−3.65)**	**28.03 (+/−3.85)**
		f	**81.39 (+/−2.179) [p = 0.0017]**	**17.61 (+/−2.06) [p = 0.0161]**
	T2DM	m	**86.30 (+/−2.35)**	34.35 (+/−2.55)
		f	**77.23 (+/−2.01) [p = 0.008]**	30.00 (+/−3.11)
	AD + T2DM	m	73.74 (+/−5.46)	34.35 (+/−2.55)
		f	70.80 (+/−3.92)	30.00 (+/−3.11)

AD = Alzheimer Disease; T2DM = Diabetes type II; m = male; f = female; MW(%) = mean value of enumerating cells in percent. Statistical Analysis was done via ANOVA and followed post−hoc scheffé test; **Bold** = $p < 0.05$.

Supplementary Table 3: Correlation between age, insulin receptor (IR) ß positive cells and peroxisome proliferator−activated receptor−γ (PPARγ) phosphorylated (p) in post−mortem human brain.

Region	R−value p−Value	Age				PPARγ (p)				IRβ			
		Control	AD	T2DM	AD + T2DM	Control	AD	T2DM	AD + T2DM	Control	AD	T2DM	AD + T2DM
Cortex	age	–	–	–	–	0.2	0.276	**0.558**	0.019	−0.004	−0.246	**−0.548**	**0.848**
						0.3015	0.927	**0.0032**	0.9238	0.9847	0.1999	**0.0112**	**< 0.0001**
	PPARγ (p)	0.2	0.276	**0.558**	0.019	–	–	–	–	−0.028	−0.383	0.032	0.102
		0.3015	0.927	**0.0032**	0.9238					0.9024	0.1811	0.9270	0.6829
	IRβ	−0.004	−0.246	**−0.548**	**0.848**	−0.028	−0.383	0.032	0.102	–	–	–	–
		0.9847	0.1999	**0.0112**	**< 0.0001**	0.9024	0.1811	0.9270	0.6829				
dorsal Hippocampus	age	–	–	–	–	−0.160	0.584	**0.591**	−0.349	−0.150	−0.198	0.243	−0.391
						0.4027	0.0205	**0.0066**	0.1125	0.5087	0.3464	0.3062	0.1219
	PPARγ (p)	−0.160	**0.584**	0.591	−0.349	–	–	–	–	−0.056	**−0.743**	−0.135	0.120
		0.4027	**0.0205**	0.0066	0.1125					0.8071	**0.0113**	0.6524	0.6774
	IRβ	−0.150	−0.198	0.243	−0.391	−0.056	−0.743	−0.135	0.120	–	–	–	–
		0.5087	0.3464	0.3062	0.1219	0.8071	0.0113	0.6524	0.6774				
ventral Hippocampus	age	–	–	–	–	**−0.578**	**0.877**	−0.099	0.409	−0.083	−0.248	**0.776**	−0.352
						0.0065	**< 0.0001**	0.6401	0.1043	0.7314	0.1885	**0.0112**	0.1289
	PPARγ (p)	−0.578	0.877	−0.099	0.409	–	–	–	–	−0.290	−0.027	−0.554	0.081
		0.0065	< 0.0001	0.6401	0.1043					0.3450	0.9325	0.1267	0.7876
	IRβ	−0.083	−0.248	0.776	−0.352	−0.290	−0.027	−0.554	0.081	–	–	–	–
		0.7314	0.1885	0.0112	0.1289	0.3450	0.9325	0.1267	0.7876				
all brain regions	age	–	–	–	–	−0.101	**0.529**	**0.323**	0.031	−0.078	−0.221	**0.482**	**−0.356**
						0.3747	**0.0002**	**0.0066**	0.8000	0.5372	0.0428	**0.0009**	**0.0062**
	PPARγ (p)	−0.101	**0.529**	**0.323**	0.031	–	–	–	–	−0.090	−0.327	−0.176	0.165
		0.3747	**0.0002**	**0.0066**	0.8000					0.5067	0.048	0.3225	0.2632
	IRβ	−0.078	**−0.221**	**0.482**	**−0.356**	−0.090	**−0.327**	−0.176	0.165	–	–	–	–
		0.5372	**0.0428**	**0.0009**	**0.0062**	0.5067	**0.048**	0.3225	0.2632				

AD = Alzheimer Disease; T2DM = Diabetes type II. Statistical Analysis was done via ANOVA and followed post−hoc scheffé test; **Bold** = $p < 0.05$.

Supplementary Table 4: Ratio between Insulin receptor (IR) β and amount of neurons in postmortem brains; AD = Alzheimer disease; T2DM = type 2 diabetes mellitus.

Region	Group	Ratio (IRβ positive cells/ amount of neurons)
prefrontal Cortex	Control	1.47
	AD	1.14
	AD + T2DM	1.58
	T2DM	1.86
dorsal Hippocampus	Control	1.85
	AD	1.38
	AD + T2DM	1.29
	T2DM	1.05
ventral Hippocampus	Control	2.01
	AD	1.24
	AD + T2DM	1.35
	T2DM	1.88

MANUSCRIPT III

Journal of Neural Transmission

"70th Birthday Prof. Riederer"-Different effects of soluble and aggregated Amyloid β42 on gene/ protein expression and enzyme activity involved in insulin and APP pathways

--Manuscript Draft--

Manuscript Number:	
Full Title:	"70th Birthday Prof. Riederer"-Different effects of soluble and aggregated Amyloid β42 on gene/ protein expression and enzyme activity involved in insulin and APP pathways
Article Type:	Full paper
Keywords:	Alzheimer´s dementia, soluble/aggregated Aβ42, GSK3β, insulin receptor, MAO-B
Corresponding Author:	Jasmin Bartl Universityhospital of Wuerzburg Wuerzburg, GERMANY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universityhospital of Wuerzburg
Corresponding Author's Secondary Institution:	
First Author:	Jasmin Bartl
First Author Secondary Information:	
Order of Authors:	Jasmin Bartl
	Andrea Meyer
	Svenja Brendler
	Peter Riederer
	Edna Grünblatt
Order of Authors Secondary Information:	
Abstract:	Although Alzheimer´s dementia (AD) is not anymore characterised just by the accumulation and deposition of amyloid beta (Aβ) peptides and hyperphosphorylation of tau proteins within the brain, excessive Aβ42 deposition is still considered to play a major role in AD. Aβ are able to adopt many differently aggregate forms, including amyloid fibrils as well as nonfibrillar structures (soluble Aβ42 oligomers). It is not well established which Aβ42 state is most responsible for AD or why. We wanted to verify, which effects Aβ42 oligomers and aggregated peptides have on gene expression, protein level and enzyme activity of insulin and amyloid precursor protein (APP) pathways in vitro. Human neuroblastoma cells (SH-SY5Y) were treated with varying concentrations of soluble and aggregated Aβ42. Treatment effects on ß-secretase (BACE), glycogen synthase kinase 3α (GSK3α), glycogen synthase kinase 3ß (GSK3ß), phosphatidylinisitol-3 kinase (PI-3K), insulin degrading enzyme (IDE), insulin receptor substrate 1 (IRS1), insulin receptor (INSR) and monoamine oxidase B (MAO-B) was investigated via quantitative-PCR, western blot, ELISA and enzyme activity assay. We could find different effects of soluble and aggregated peptides especially on gene/ protein expression of GSK3β and INSR and on GSK3β and MAO-B activity. Soluble peptides showed significant effects leading to increased gene expression and protein amount of GSK3β and to decreased level of gene and protein expression of INSR. MAO-B activity was enhanced after treatment with aggregated peptides and strongly inhibited after soluble Aβ42 treatment. Our data might provide insights into selective effects of specific forms of Aß42 aggregates in AD.

Powered by Editorial Manager® and Preprint Manager® from Aries Systems Corporation

Introduction

Alzheimer´s dementia (AD) is not anymore characterised just by the accumulation and deposition of amyloid beta (Aβ) peptides and hyperphosphorylated tau proteins within the brain; in the mean time, it is also characterised by synaptic loss (Small, 2004), imbalanced metabolism (Schindowski et al., 2008; Grünblatt et al., 2010), abnormal protein cross-linking (Munch et al., 1998; Wang et al., 2008), and disturbance of the insulin signalling pathway (Hoyer et al., 1994; de la Monte et al., 2009; Riederer et al., 2010). Although more and more factors are found to be involved in AD, excessive $A\beta_{42}$ deposition is still considered to play a major role in AD. However, the mechanisms that contribute to abnormal $A\beta_{42}$ accumulation are not fully understood, both increased production and decreased degradation have been observed (Crouch et al., 2008). $A\beta_{42}$ are able to adopt many differently shaped aggregates including amyloid fibrils (Glabe, 2008) as well as nonfibrillar aggregates that are also termed soluble $A\beta_{42}$ “oligomers”(Haass and Selkoe, 2007). It is not well established which Aβ state is most responsible for AD or why. Recent evidence strongly implicates $A\beta_{42}$ oligomers as the proximal pathogenic trigger (De Felice et al., 2008). These oligomers are markedly elevated in the brain and cerebrospinal fluid of postmortem AD patients and appear to play a critical role in the synaptic failure and memory deficits of early AD (Klyubin et al., 2008). It is therefore important to determine directly whether neurons exposed to oligomers undergo pathological changes characteristic of AD brain. In the current study, we sought to verify the effects of both oligomers and aggregated $A\beta_{42}$ peptides on gene expression, protein level and enzyme activity *in vitro*. The cell culture model chosen for this investigation was the human SH-SY5Y neuroblastoma cell line. These cells show the most common neurobiochemical (e.g. enzymes like dopamine β-hydroxylase, acetylcholintransferase, etc. and neurotransmitter like dopamine, adrenaline, acetylcholine, GABA) and neurobiological factors (e.g. dopamine transporter and receptors, GABAergic receptors, etc.) (Biedler et al., 1978; Ross et al., 1983) and can be perfectly used as an experimental model to study effects of $A\beta_{42}$ oligomers and aggregated $A\beta_{42}$ peptides. Our main focus lay on treatment effects on β-secretase (BACE), glycogen synthase kinase 3α (GSK3α), glycogen synthase kinase 3β (GSK3β), phosphatidylinisitol-3 kinase (PI-3K), insulin degrading enzyme (IDE), insulin receptor substrate 1 (IRS1), insulin receptor (INSR) and monoamine oxidase B (MAO-B). They are all already known to be involved in the pathology of

AD: BACE is the first protease in the processing of amyloid precursor protein (APP) leading to the production of Aβ_{42} in the brain, therefore it is one recent target for therapeutic treatment of AD (Ghosh et al., 2011). Over-activity of GSK3 accounts for memory impairment, tau hyperphosphorylation, increased Aβ production and local plaque-associated microglial-mediated inflammatory responses; all of which are hallmark characteristics of AD (reviewed in (Hooper et al., 2008)). PI-3K has been identified as second messenger system providing anti-apoptotic signal to various cell types (Brunet et al., 2001). Additionally, neuroprotection against extracellular Aβ_{42} toxicity is dependent on PI-3K pathway (Zhang et al., 2003; Lesne et al., 2005). IDE is a metalloprotease that apart from insulin also binds and degrades other substrates, including Aβ_{42}, therefore reduced IDE activity diminishes Aβ clearance and contributes to the formation of senile plaques (Mukherjee et al., 2000). Not only for IDE insulin and Aβ_{42} are counterparts, in fact Aβ_{42} peptides directly compete with insulin for binding to the INSR (de la Monte et al., 2009) and INSR dysfunction impairs cellular clearance of neurotoxic oligomeric Aβ_{42} (Zhao et al., 2009). MAO-B activity is significantly increased in AD patients (Gotz et al., 1998; Grünblatt et al., 2005) and inhibition of MAO-B has a neuroprotective effects for AD (reviewed in (Riederer et al., 2004).

Material and Methods

Cell culture

SH-SY5Y cells were bought from European Collection of Cell Cultures and grown in buffered Dulbecco`s modified Eagle medium (DMEM/F-12) (Pan Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum and 0.1% gentamycin (50 mg/ml) (Life technologies, Darmstadt, Germany) in a humidified incubator (5% CO_2) at 37°C.

Preparation of soluble and aggregated Aβ_{42}

The preparation of the Aβ_{42} peptide was modified according to Dahlgren (Dahlgren et al., 2002). 500mg of freeze-dried Aβ_{42} (Millipore, Schwalbach, Germany) was dissolved in 1,1,1,3,3,3 hexafluoroisopropanol (HFIP) to a concentration of 1mM and incubated for one hour at room temperature. Subsequently, the HFIP was removed by using a vacuum centrifuge and the peptide film

was dissolved in dry dimethyl sulfoxide (DMSO) to a final concentration of 5mM. For aggregated $A\beta_{42}$ peptides, the solution was diluted with 10mM HCl and stored for 7 days in the incubator at 37°C. The test concentrations of both the soluble and the aggregated $A\beta_{42}$ were finally diluted with normal cell culture medium.

Cell treatment with $A\beta_{42}$

For western blot (WB), enzyme linked immunosorbent assay (ELISA), fluorescent ELISA and quantitative real-time polymerase chain reaction (QRT-PCR) , SH-SY5Y cells were treated with varying concentrations of soluble and aggregated $A\beta_{42}$ (see preparation above) (0, 5, 1, 2,5 and 10µM), or purified DMSO or HCl as a control, by adding it in the right concentration to the culture medium. The reaction was stopped after 48 h and cells were trypsinized for further investigations.

Cytotoxicity

To determine a possible toxic effect of $A\beta_{42}$ on the cells, the CytoTox-Glo™ test from Promega (Mannheim, Germany) was performed. For testing the cytotoxicity effect, 10.000cells/ml per well were cultivated for 24 hours at 37°C in 5% CO_2 saturation in the incubator, so that the cells could adhere to the bottom of the wells. Afterwards cells were treated with soluble or aggregated $A\beta_{42}$ in a concentration of 0.1, 0.5, 1, 2, 3, 5, 10, 20, 50 and 100 µM. Reaction was measured 48h later.

RNA extraction and QRT-PCR

Total RNA was extracted from SH-SY5Y cells using the RNeasy Plus MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany). QRT-PCR was performed using an iCycler iQ™ Real Time PCR Detection System (Bio-Rad) and the SYBR-Green detection method. The QRT-PCR reaction was optimized according to the manufacturer's instructions. QuantiTech Primer assays for β-actin (*ACTB*; QT00193473), glyceraldehyde-3-phosphate dehdroge-nase (*GAPDH,* QT01192646*)*, ribosomal protein L13A (*RPL13A*; QT00089915), peptidylprolyl isomerase A (*PPIA*, QT01866137), β-secretase (*BACE*, QT00084777), glycogen synthase kinase 3α (*GSK3α*, QT00075306), glycogen synthase kinase 3β (*GSK3β*, QT00057134), phosphatidylinisitol-3

kinase (*PI-3K*, QT00035175), insulin degrading enzyme (*IDE*, QT00080773), insulin receptor substrate 1 (*IRS1*, QT00074144) and insulin receptor (*INSR*, QT00082810) were purchased from Qiagen (Hilden, Germany). *ACTB, GAPDH, RPL13A* and *PPIA* were used for normalization according to GeNorm (Vandesompele et al., 2002). The amplified transcripts were quantified using the comparative threshold cycle (Ct) analyzed using the BioRad iCycler iQ program.

Western blot

Western blotting was used to detect changes in the protein levels of Insulin receptor (IR) β subunit in SH-SY5Y cells. To determine total protein amount of each sample, Bradford protein assay was conducted (Sigma-Aldrich, Schorndorf, Germany). Samples (50µg of total protein, denaturated with dithiothreitol) were separated on 4-12% Bis-Tris gel and transferred onto nitrocellulose membranes (Life technologies, Darmstadt, Germany). Protein of interest was detected using its specific antibodies: rabbit anti-IRβ (antibody) (1:250, Santa Cruz, Heidelberg, Germany). Following blocking the membrane for one hour at room temperature (5% blocking milk in TBS-Tween) and afterwards incubation with primary antibody at 4°C overnight, membranes were incubated with HRP conjugated secondary antibody (1:15.000, Sigma-Aldrich, Schorndorf, Germany) for one hour at room temperature. The IRβ protein immunoreactive bands were detected using the enhanced chemiluminescence (ECL) detection plus system from GE Healthcare (Freiburg, Germany) and quantified using ImageJ software without modifying picture properties (e.g. gain, colour or contrast). For comparison the values of β-actin, as a house keeping protein, were used. The membranes were stripped to remove the attached antibodies. It was followed by one-hour incubation in 5% BSA blocking solution at room temperature. The murine β-actin antibody (1:5000, Santa Cruz, Heidelberg, Germany) was already linked with HRP and was detected and quantified like IRβ protein.

Enyzme linked immunosorbent assays

The Invitrogen (Darmstadt, Germany) GSK-3β kit is a solid phase sandwich enzyme linked immunosorbent assay (ELISA) and was used to detect and quantify the level of total GSK-3β protein and of GSK-3β protein phosphorylated at serine residue 9. Samples were prepared according to

manual. Detected protein amount was normalized with total protein amount of used samples, as detected via Bradford protein assay (Sigma-Aldrich, Schorndorf, Germany).

Enzyme activity

MAO-B enzyme activity was measured by using amplex red monoamine oxidase assay kit (Life technologies, Darmstadt, Germany). 50µg of total protein (detected via Bradford protein assay) of homogenized SH-SY5Y cells was diluted in reaction buffer and varying concentrations of soluble and aggregated $A\beta_{42}$ (see preparation above) (0.5, 1, 2.5 and 10µM) were added. After 30 minutes incubation at 37 °C, 100µl substrate mix was added and again incubated for 1 hour at 37°C. The endpoint measurement was done in a fluorescence multi plates meter (Novostar) at an excitation of 542 nm and an emission of 590 nm.

Statistical analysis

Statistical analyses of the WB, ELISA and QRT-PCR data were performed using StatView for Windows (SAS Institute Inc., version 5). Statistical tests included analysis of variance (ANOVA) with post-hoc Scheffé Test. Significance was set as $p<0.05$. For a better comparison of the data, we normalized the results and converted the data into percentages. The control group was set as 100%. The results represent at least 3 repeated experiments with around 4 internal repeats.

Results

Soluble and aggregated species of $A\beta_{42}$ were prepared as described above and controlled via electron microscopy (shown in supplementary figure 1). Avoiding neural cell death, we tested a wide range of $A\beta_{42}$ µM doses (0-100µM) before cell treatment and used just the nontoxic range from 0-10µM for this study. Concentration of over 20µM aggregated $A\beta_{42}$ showed significant cell toxicity, while for soluble form only a trend to toxicity was observed (supplementary figure 2).

Gene expression

BACE, GSK3α, GSK3β, PI-3K, IDE, IRS-1 and *INSR* were the investigated genes. After gene expression analysis we could detect two genes with significant differences between treated and

untreated cells. *GSK3β* was significantly up-regulated after cell treatment with 1 and 2µM (+362% $p<0.05$; +357 $p<0.05$) of soluble $A\beta_{42}$ and we could detect a tendency of higher gene expression after treatment with 1, 2, 5 and 10µm of the aggregated $A\beta_{42}$ (Table 1). The second gene revealing statistically significant results was the *INSR*. Treatment with 5 and 10µM of soluble peptides tended to result in a lower *INSR* gene expression compared to untreated cells while on the other hand, treatments with 1µM of the aggregated peptides tended to result in a higher expression and treatment with 5 and 10µM even led to a significant overexpression of *INSR* (+183 $p<0.05$; +201 $p<0.05$; Table 1). Based on these results we further investigated the protein expression of these two genes in order to examine whether the differences influence gene transcription.

Protein level

After SH-SY5Y cells were treated with all doses of soluble $A\beta_{42}$, significantly more GSK3β protein levels (30-45%) was detectable compared to the control cells ($p<0.01$, figure 1a), which confirm the gene expression data of *GSK3β.* Treatments with aggregated peptides did not show any significant effects on GSK3β total protein levels in non of the used doses (figure 1A). Because GSK3β is a kinase enzyme, that when phosphorylated at serine residue 9 becomes inactive, we investigated the phosphorylated form of this protein to affirm whether it is more or less active after $A\beta_{42}$ treatment to the cells. We calculated the ratio between total amount of GSK3β protein and its phosphorylated form, which represents the enzyme activity (figure 1B). Remarkably, not only the total protein amount of GSK3β was statistically significantly increased after treatment with soluble peptides, but there was also a significant detectable enhancement of the enzyme activity under this condition (145%-231% more activity in all used dosages compared to untreated cells; $0.01<p<0.05$; figure 1B). On the other hand the enzyme activity did not alter after treatment with aggregated peptides (figure 1B).

The gene expression data of *INSR* demonstrated already a tendency for reduced expression after treatment with soluble peptides (table 1), which resulted in significantly decreased total amount of IRβ after treatment with 0.5, 1 and 2µM of soluble $A\beta_{42}$ (50%-60% less total IRβ protein amount compared to untreated cells, $p<0.05$; figure 2). On the other hand, in the gene expression analysis a significant overexpresion of *INSR* after treatment with aggregated peptides was observed (table 1),

which was confirmed only at 1µM aggregated peptide treatment for the total IRβ protein expression. At 1µM of aggregated $A\beta_{42}$ we could detect 60% more IRβ compared to control cells ($p<0.05$; figure 2).

Enzyme activity

MAO B enzyme activity was significantly reduced after cell homogenate treatment with 1, 2, 5 and 10µM of soluble $A\beta_{42}$ peptides (18%-30% less activity, $p<0.01$; figure 3). In contrast the aggregated peptides enhanced the enzyme activity with significant results at 1, 2 and 5µM of aggregated peptide (30%-40% more activity compared to control; $p<0.01$; figure 3).

Discussion

AD is one of the most common form of dementia in the elderly (Blennow et al., 2006) and although increasing factors are found to be involved in AD, excessive $A\beta_{42}$ deposition is still considered to play a major role in this disease. $A\beta_{42}$ is derived from the APP by the action of two aspartyl proteases, β- and γ-secretases. So accrued $A\beta_{42}$ molecules tend to aggregate to form soluble oligomers, protofibrils, and β-amyloid fibrils, which have been suggested to cause neuronal dysfunction in the brains of AD patients (reviewed in (Ono and Yamada, 2011)). However, it is not well established which $A\beta_{42}$ state is most responsible for AD or why. In this study we verified, which effects $A\beta_{42}$ oligomers and aggregated $A\beta_{42}$ peptides have on gene expression, protein level and enzyme activity of the insulin and APP pathways *in vitro*. In particular, we have observed a strong influence of the soluble form of $A\beta_{42}$ peptides on GSK3β gene expression as well as on its protein level and activity. It has been already postulated that GSK3β activity might exert a central role in the development of AD (Hooper et al., 2008). GSK3β activity was implicated in tau phosphorylation, APP processing, $A\beta_{42}$ production and neurodegeneration (Balaraman et al., 2006; Hooper et al., 2008). Our finding of increased GSK3β gene/protein expression and activity points to the fact, that GSK3β might be a crucial element in the $A\beta_{42}$-triggered molecular cascade leading to potential neurodegeneration. The observed effect of $A\beta_{42}$ oligomers on GSK3β might be mediated by interaction with different receptors, but mainly with the interaction of INSR. It was already shown that $A\beta_{42}$ oligomers interact with neuronal INSR to cause

impairments of the receptor expression and function (Townsend et al., 2007; Zhao et al., 2009). Normally, activation of INSR leads to the phosphorylation of PI 3-kinase, which leads to an activation of AKT. Once active, AKT enters the cytoplasm where it leads to the phosphorylation and inactivation of GSK3β (Jolivalt et al., 2008). Thus, although normal INSR activity helps to defend against the accumulation of toxic $A\beta_{42}$ oligomers, the INSR themselves are vulnerable to oligomer-initiated dysfunction. The presented data indicate the fact that this relationship has the potential to generate an insidious pathogenic loop. We could observe a significant reduction of INSR gene and protein expression after SH-SY5Y treatment with soluble $A\beta_{42}$ oligomers compared to untreated cells. Consistent with the actual finding reported here, our group could already report that in AD patient less INSR were found especially in the hippocampal region of the brain (Bartl et al., 2012). Not only an impairment of INSR leads to an activation of GSK3β as explained before, also oxidative stress can enhance the activity of this kinase (Hernandez and Avila, 2008). In 2004, Riederer et al. already reviewed the link between oxidative stress and AD, especially regarding the increased MAO-B activity (Riederer et al., 2004). MAO-B is a flavin containing enzyme localized in the outer mitochondrial membrane and is responsible for the oxidative deamination of neurotransmitters (noradrenaline, dopamine and serotonin) and exogenous amines. During its catalytic activity it produces hydrogen peroxide formed in the reaction, which is a possible source for oxidative stress. Highlighting more the possible link between $A\beta_{42}$, GSK3 and MAO-B we analyzed the effect of soluble and aggregated peptides on MAO-B enzyme activity. Interestingly, the soluble oligomers inhibited the enzyme activity significantly, but the aggregated forms of $A\beta_{42}$ peptides, which are more similar to the amyloid plaques within the brain, enhanced the MAO-B activity in a dose dependent manner. Although we could find an inhibition of MAO-B activity after soluble peptide treatment, it cannot be concluded that these oligomers might be neuroprotective. An increased MAO-B activity was found in senile plaques (Saura et al., 1997) and in platelets of AD patients (Gotz et al., 1998), therefore our results of enhanced MAO-B activity following treatment with aggregated while not with soluble $A\beta_{42}$ peptides, might indicate the involvement of the fibrillar structures of $A\beta_{42}$ in the oxidative stress cascade known to play an important role in AD (Grünblatt et al., 2005; Grünblatt et al., 2010). To our knowledge, this is the first report indicating the direct influence of soluble or aggregated

peptides on MAO-B activity *in vitro.* Our data affirm that there has to be a neurobiochemical link between MAO-B and Aβ_{42}. In conclusion, our results suggest that Aβ_{42} oligomers seem to be more involved in cellular mechanisms linked to AD, except in enhancement of MAO-B activity, than the aggregated Aβ_{42} peptides, which is confirmed with the actual literature. AD seem to involve numerous mechanisms such as amyloid plaque formation, tau hyperphoshprylation, apoptosis, impairment of insulin signaling pathway and oxidative stress, causing neurodegeneration, which means that further investigations are necessary to enlighten the molecular mechanism of AD pathogenesis.

Acknowledgments and Funding

Funding for this study was partially provided by the "Alzheimer Forschungs Initiative (AFI)", Düsseldorf, Germany; the AFI had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. We thank Miryame Hofmann for her excellent technical assistance and also Dr. Zoya Marinova for her great and excellent support.

References

Balaraman Y, Limaye AR, Levey AI, Srinivasan S (2006) Glycogen synthase kinase 3beta and Alzheimer's disease: pathophysiological and therapeutic significance. Cell Mol Life Sci 63:1226-1235.

Bartl J, Monoranu CM, Wagner AK, Kolter J, Riederer P, Grunblatt E (2012) Alzheimer's disease and type 2 diabetes: Two diseases, one common link? World J Biol Psychiatry.

Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS (1978) Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res 38:3751-3757.

Blennow K, de Leon MJ, Zetterberg H (2006) Alzheimer's disease. Lancet 368:387-403.

Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11:297-305.

Crouch PJ, Harding SM, White AR, Camakaris J, Bush AI, Masters CL (2008) Mechanisms of A beta mediated neurodegeneration in Alzheimer's disease. Int J Biochem Cell Biol 40:181-198.

Dahlgren KN, Manelli AM, Stine WB, Jr., Baker LK, Krafft GA, LaDu MJ (2002) Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. J Biol Chem 277:32046-32053.

De Felice FG, Wu D, Lambert MP, Fernandez SJ, Velasco PT, Lacor PN, Bigio EH, Jerecic J, Acton PJ, Shughrue PJ, Chen-Dodson E, Kinney GG, Klein WL (2008) Alzheimer's disease-type neuronal tau hyperphosphorylation induced by A beta oligomers. Neurobiol Aging 29:1334-1347.

de la Monte SM, Longato L, Tong M, Wands JR (2009) Insulin resistance and neurodegeneration: roles of obesity, type 2 diabetes mellitus and non-alcoholic steatohepatitis. Curr Opin Investig Drugs 10:1049-1060.

Ghosh AK, Brindisi M, Tang J (2011) Developing beta-secretase inhibitors for treatment of Alzheimer's disease. J Neurochem 120 Suppl 1:71-83.

Glabe CG (2008) Structural classification of toxic amyloid oligomers. J Biol Chem 283:29639-29643.

Gotz ME, Fischer P, Gsell W, Riederer P, Streifler M, Simanyi M, Muller F, Danielczyk W (1998) Platelet monoamine oxidase B activity in dementia. A 4-year follow-up. Dement Geriatr Cogn Disord 9:74-77.

Grünblatt E, Bartl J, Riederer P (2010) The link between iron, metabolic syndrome, and Alzheimer's disease. J Neural Transm 118:371-379.

Grünblatt E et al. (2005) Oxidative stress related markers in the "VITA" and the centenarian projects. Neurobiol Aging 26:429-438.

Haass C, Selkoe DJ (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol 8:101-112.

Hernandez F, Avila J (2008) The role of glycogen synthase kinase 3 in the early stages of Alzheimers' disease. FEBS Lett 582:3848-3854.

Hooper C, Killick R, Lovestone S (2008) The GSK3 hypothesis of Alzheimer's disease. J Neurochem 104:1433-1439.

Hoyer S, Muller D, Plaschke K (1994) Desensitization of brain insulin receptor. Effect on glucose/energy and related metabolism. J Neural Transm Suppl 44:259-268.

Jolivalt CG, Lee CA, Beiswenger KK, Smith JL, Orlov M, Torrance MA, Masliah E (2008) Defective insulin signaling pathway and increased glycogen synthase kinase-3 activity in the brain of diabetic mice: parallels with Alzheimer's disease and correction by insulin. J Neurosci Res 86:3265-3274.

Klyubin I, Betts V, Welzel AT, Blennow K, Zetterberg H, Wallin A, Lemere CA, Cullen WK, Peng Y, Wisniewski T, Selkoe DJ, Anwyl R, Walsh DM, Rowan MJ (2008) Amyloid beta protein dimer-containing human CSF disrupts synaptic plasticity: prevention by systemic passive immunization. J Neurosci 28:4231-4237.

Lesne S, Gabriel C, Nelson DA, White E, Mackenzie ET, Vivien D, Buisson A (2005) Akt-dependent expression of NAIP-1 protects neurons against amyloid-{beta} toxicity. J Biol Chem 280:24941-24947.

Mukherjee A, Song E, Kihiko-Ehmann M, Goodman JP, Jr., Pyrek JS, Estus S, Hersh LB (2000) Insulysin hydrolyzes amyloid beta peptides to products that are neither neurotoxic nor deposit on amyloid plaques. J Neurosci 20:8745-8749.

Munch G, Schinzel R, Loske C, Wong A, Durany N, Li JJ, Vlassara H, Smith MA, Perry G, Riederer P (1998) Alzheimer's disease--synergistic effects of glucose deficit, oxidative stress and advanced glycation endproducts. J Neural Transm 105:439-461.

Ono K, Yamada M (2011) Low-n oligomers as therapeutic targets of Alzheimer's disease. J Neurochem 117:19-28.

Riederer P, Danielczyk W, Grunblatt E (2004) Monoamine oxidase-B inhibition in Alzheimer's disease. Neurotoxicology 25:271-277.

Riederer P, Bartl J, Laux G, Grunblatt E (2010) Diabetes type II: a risk factor for depression-Parkinson-Alzheimer? Neurotox Res 19:253-265.

Ross RA, Spengler BA, Biedler JL (1983) Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J Natl Cancer Inst 71:741-747.

Saura J, Andres N, Andrade C, Ojuel J, Eriksson K, Mahy N (1997) Biphasic and region-specific MAO-B response to aging in normal human brain. Neurobiol Aging 18:497-507.

Schindowski K, Belarbi K, Buee L (2008) Neurotrophic factors in Alzheimer's disease: role of axonal transport. Genes Brain Behav 7 Suppl 1:43-56.

Small DH (2004) Mechanisms of synaptic homeostasis in Alzheimer's disease. Curr Alzheimer Res 1:27-32.

Townsend M, Mehta T, Selkoe DJ (2007) Soluble Abeta inhibits specific signal transduction cascades common to the insulin receptor pathway. J Biol Chem 282:33305-33312.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.

Wang DS, Dickson DW, Malter JS (2008) Tissue transglutaminase, protein cross-linking and Alzheimer's disease: review and views. Int J Clin Exp Pathol 1:5-18.

Zhang Y, Hong Y, Bounhar Y, Blacker M, Roucou X, Tounekti O, Vereker E, Bowers WJ, Federoff HJ, Goodyer CG, LeBlanc A (2003) p75 neurotrophin receptor protects primary cultures of human neurons against extracellular amyloid beta peptide cytotoxicity. J Neurosci 23:7385-7394.

Zhao WQ, Lacor PN, Chen H, Lambert MP, Quon MJ, Krafft GA, Klein WL (2009) Insulin receptor dysfunction impairs cellular clearance of neurotoxic oligomeric a{beta}. J Biol Chem 284:18742-18753.

Table 1: Gene expression data after SH-SY5Y treatment with soluble and aggregated Aβ_{42} peptides.

	soluble Aβ42 (µM) [mean value (%) ± standard derivate(%)]					aggregated Aβ42 (µM) [mean value(%) ± standard derivate(%)]				
Gene / Doses	0.5	1	2	5	10	0.5	1	2	5	10
BACE	97 ±20	88 ±19	96 ±19	105 ±21	103 ±22	182 ±88	129 ±37	247 ±153	108 ±47	161 ±57
GSK3α	98 ±26	79 ±20	88 ±19	69 ±13	97 ±19	96 ±12	97 ±9	105 ±12	110 ±15	115 ±14
GSK3β	149 ±37	**462 ±160**	**457 ±206**	264 ±85	220 ±57	83 ±11	*337 ±88*	*379 ±91*	*286 ±57*	*335 ±74*
PI-3K	148 ±59	145 ±40	97 ±25	86 ±20	100 ±24	114 ±43	119 ±39	101 ±29	120 ±39	111 ±28
IDE	89 ±60	81 ±52	58 ±39	83 ±59	80 ±55	77 ±24	90 ±31	91 ±32	71 ±21	115 ±41
IRS-1	36 ±99	79 ±41	65 ±21	97 ±43	129 ±62	54 ±18	114 ±57	*57 ±13*	*52 ±18*	*52 ±18*
INSR	101 ±17	70 ±11	90 ±5	*75 ±7*	76 ±14	197 ±71	*195 ±45*	154 ±49	**283 ±46**	**301 ±40**

Shown is the percentage of the mean values of gene expression of β-secretase (BACE), glycogen synthase kinase 3α (GSK3α), glycogen synthase kinase 3β (GSK3β), phosphatidylinisitol-3 kinase (PI-3K), insulin degrading enzyme (IDE), insulin receptor substrate 1 (IRS1), insulin receptor (INSR) and monoamine oxidase B (MAO-B).measured by real time PCR. Reference value (100%) is control group with untreated SH-SY5Y cells. For the statistical analysis ANOVA, post-hoc Scheffé with a specified significance level of p <0.05 was used. **BOLD= 0.05 < p <0.01**, *italic = 0.1 < p <0.05* versus control.

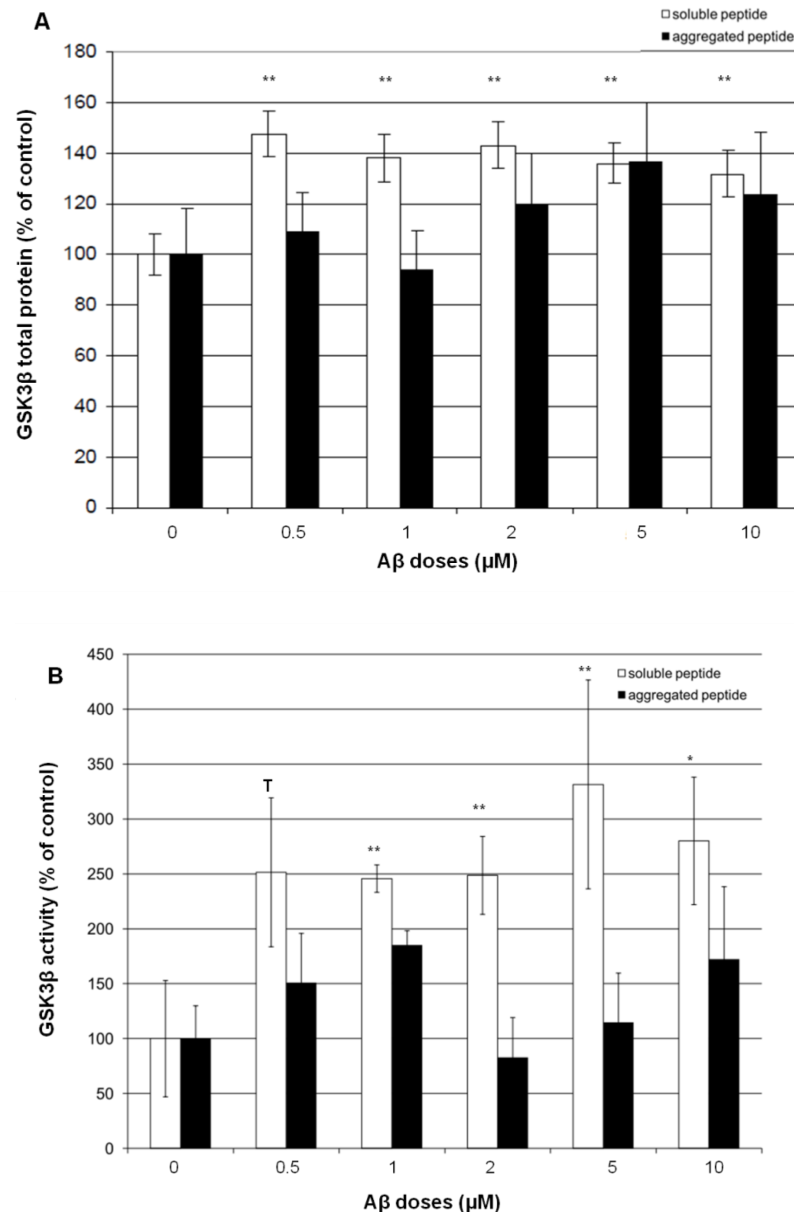


Figure 1: **Total protein amount of glycogen synthase kinase 3β (GSK3β) and ratio of phosphorylated GSK3ß / total GSK3ß after SH-SY5Y treatment with soluble and aggregated Aβ$_{42}$ peptides.** A Shown is the percentage of the mean values of total amount of the protein measured by a sandwich ELISA. B Shown is the percentage of the mean values of total amount of the protein and of phosphorylated protein amount measured by a sandwich ELISA. Reference value (100%) is control group with untreated SH-SY5Y cells. For the statistical analysis ANOVA, post-hoc Scheffé with a specified significance level of $p < 0.05$ was used. *= $p < 0.05$, ** = $p < 0.01$ versus control. The sample size was n = 6-12.

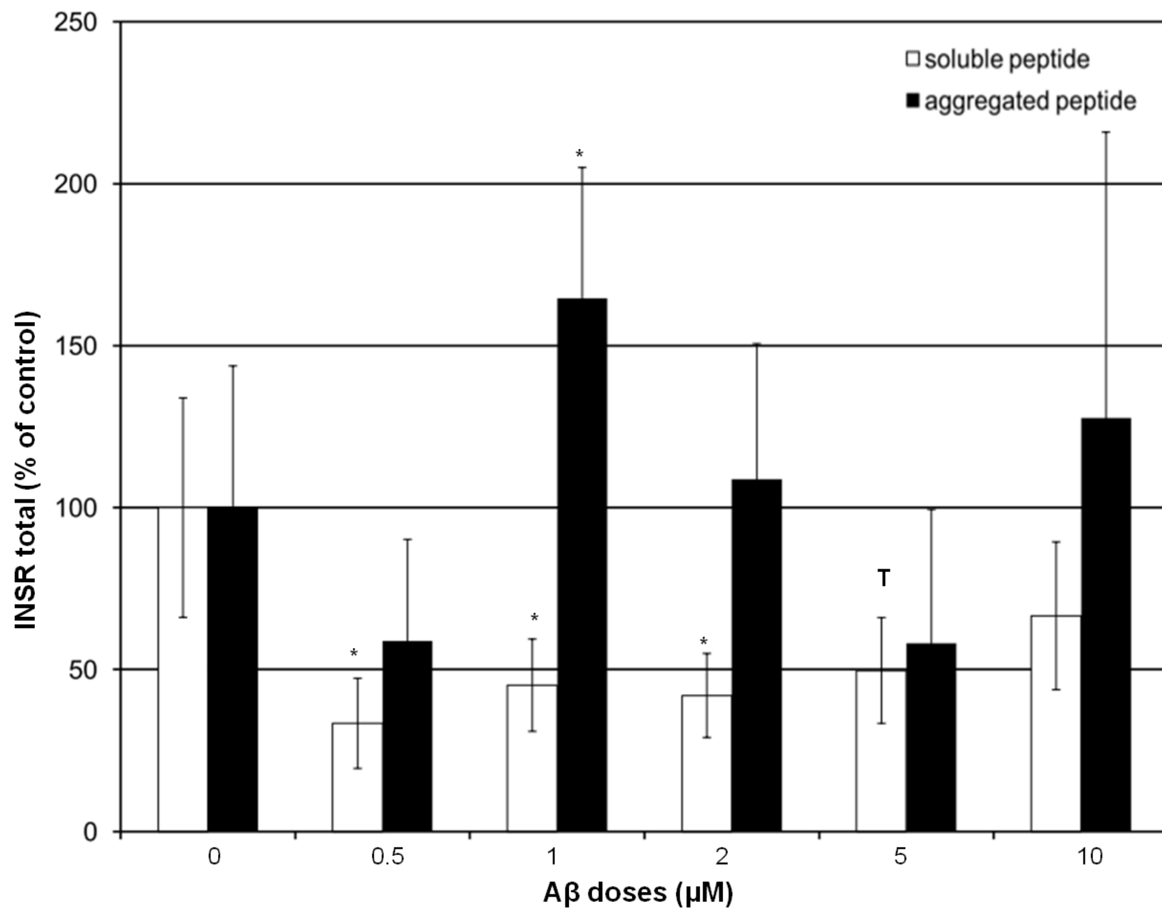


Figure 2: **Total protein amount of insulin receptor (INSR) after SH-SY5Y treatment with soluble and aggregated Aβ$_{42}$ peptides.** Shown is the percentage of the mean values of total amount of the protein measured by western blot. Reference value (100%) is control group with untreated SH-SY5Y cells. For the statistical analysis ANOVA, post-hoc Scheffé with a specified significance level of $p < 0.05$ was used. *= $p < 0.05$, ** = $p < 0.01$ versus control. The sample size was n = 6-12.

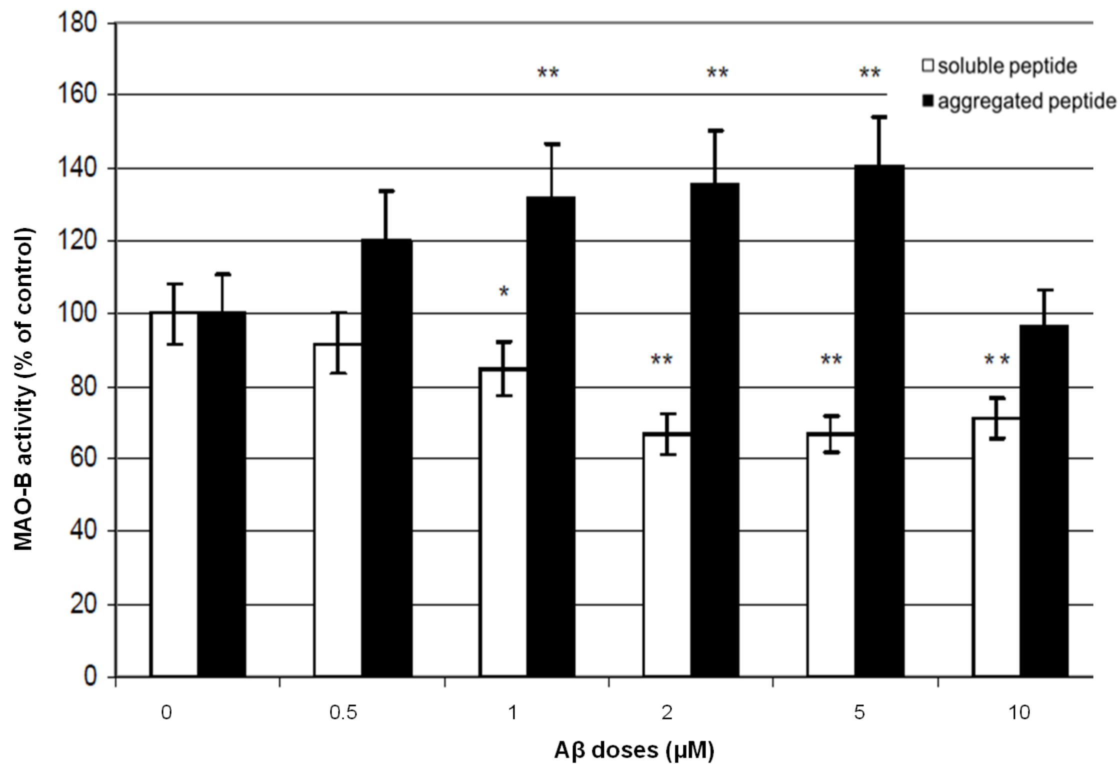
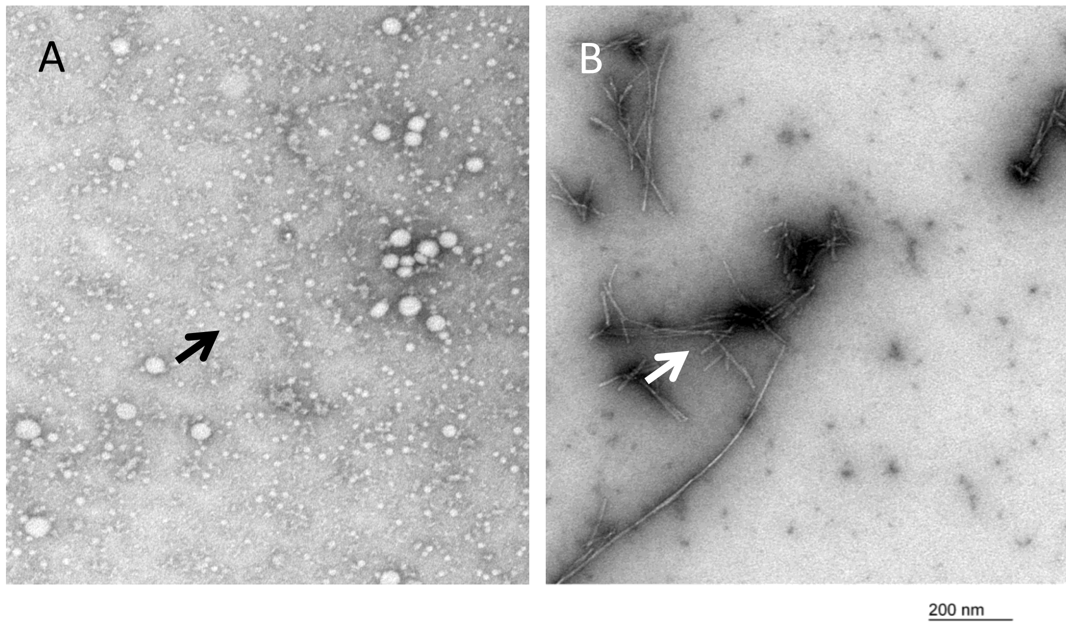
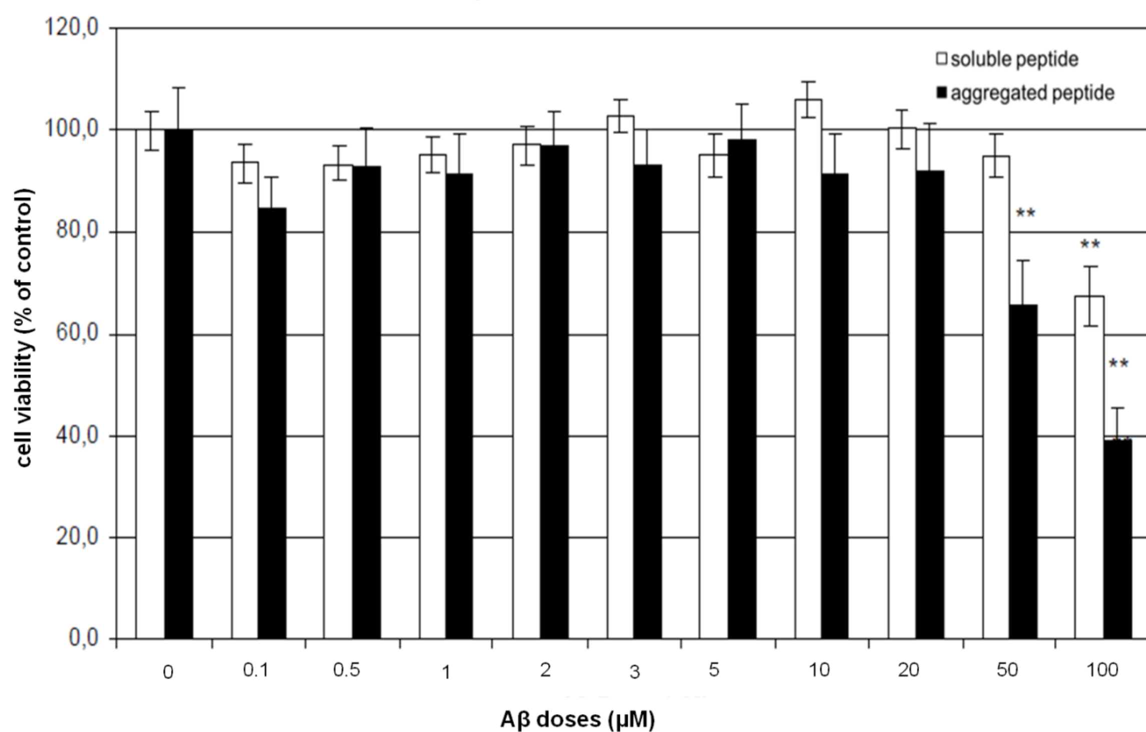


Figure 3: **Monoamino oxidase B (MAO-B) activity after SH-SY5Y treatment with soluble and aggregated Aβ$_{42}$ peptides**. Shown is the percentage of the mean values of enzyme activity measured by an amplex red monoamine oxidase assay. Reference value (100%) is control group with untreated SH-SY5Y cells. For the statistical analysis ANOVA, post-hoc Scheffé with a specified significance level of $p < 0.05$ was used. *= $p < 0.05$, ** = $p < 0.01$ versus control. The sample size was n = 20-38.



Supplementary Figure 1: **Electron microscope pictures of soluble and aggregated Aβ_{42} peptides**. 5µl of samples were spotted on a glow discharged, carbon-coated grid and incubated for 5 min, washed with distilled water, fixed with 2.5% glutaraldehyde, stained with a 1% (w/v) aqueous uranyl acetate solution, and examined using a transmission electron microscope. 20.000 fold magnification. A. arrow points to soluble Aβ_{42} peptides; B. arrow points to aggregated Aβ_{42} peptides.



Supplementary figure 2: **Cytotoxicity of soluble and aggregated $A\beta_{42}$ peptides.** Shown is the percentage of cell viability compared of control group of SH-SY5Y after 48h treatment with soluble or aggregated $A\beta_{42}$ peptides. The number of viable cells is calculated from the substraction of the dead cells of the total cell number. Statistical analysis of cytotoxicity was performed using ANOVA post-hoc Scheffé. ** = $p < 0.01$ versus control. The sample size was n=13-22.

DETAILED METHODS

MANUSCRIPT I

Subjects

Subjects of the present study were from the VITA (Vienna Transdanube Aging) study which was described previously in greater detail [88]. The VITA study investigated the residents of two Viennese districts born between May 1925 and June 1926 (i.e., aged 75 years at inclusion). Data refer to the total cohort at baseline recruitment of 606 individuals who completed physical health check, questionnaires for education, psychosocial activities and neuropsychological examination. The 1st follow-up after 30 months was possible for 476 subjects. Thirty-eight out of the 606 subjects deceased between baseline and the follow-up investigation after 30 months, 70 subjects refused to participate in the follow-up investigation and 10 subjects were willing only to take part in a telephone-interview. The 2nd follow-up after 60 months was possible for 362 subjects. 81 subjects out of the 606 participants at baseline deceased between baseline and the 2nd follow-up investigation, 92 subjects refused to take part again in the follow-up investigation, 68 subjects were willing only to take part in a telephone-interview or a house visit providing only minimal information and in three subjects no contact was possible to establish. A diagnosis of AD was established applying the NINCDS-ADRDA criteria at all-time points [89]. Additional information was obtained in all cases for relevant serum parameters such as cortisol and glucose level and T2DM was diagnosed according to the guide line of the world health organisation. All participants passed through a consensus conference with regard to the diagnoses of possible or probable AD. The final diagnosis was made by an experienced geronto-psychiatrist. The number of AD cases given in the total sample is the sum of all possible and probable cases in the whole cohort (Table 2). The VITA study was carried out with the permission of the Ethics Committee of the City of Vienna, Austria and each participant gave a written informed consent.

Table 2: Demographic information of the Vienna Transdanube Aging (VITA) study cohort.

examination	parameter	total sample	with *IDE* genotypes			
			AD		T2DM	
			no	yes	no	yes
baseline	**sex** male / female	247 / 359	232 / 321	5 / 13	194 / 281	44 / 53
	***APOE* ε4** no / yes	469 / 124	429 / 116	13 / 4	365 / 103	78 / 17
	BMI mean ± SD	27.1 ± 3.87	27.14 ± 3.81	24.82 ± 3.29	26.93 ± 3.66	27.76 ± 4.42
1st follow-up	**sex** male / female	193 / 305	150 / 239	35 / 46	147 / 242	42 / 49
	***APOE* ε4** no / yes	383 / 103	310 / 74	52 / 26	298 / 85	72 / 16
	BMI mean ± SD	27.14 ± 3.91	27.32 ± 3.84	26.22 ± 3.95	26.88 ± 3.67	28.18 ± 4.67
2nd follow-up	**sex** male / female	163 / 267	107 / 181	48 / 60	124 / 207	35 / 45
	***APOE* ε4** no / yes	327 / 93	229 / 56	73 / 33	249 / 78	65 / 13
	BMI mean ± SD	26.75 ± 4.02	27 ± 4.15	25.92 ± 3.55	26.59 ± 3.88	27.14 ± 4.34

Abbreviations used: AD = Alzheimer's disease; BMI = body mass index; SD = standard deviation; T2DM = Type 2 diabetes mellitus.

DNA extraction

DNA was prepared from 2 ml EDTA-blood by the standard procedure of proteinase K. 2 ml blood was mixed with 2 ml of lysis buffer (0.32 M sucrose 10 mM Tris–HCl, pH 7.5, 1% Triton x-100, 5 mM $MgCl_2$), vortexed and centrifuged at 13,000 × g for 20 s. The pellet was then resuspended in 4 ml PBS and centrifuged at 13,000 × g for another 20 s, followed by resuspension in 2 ml buffer with non-ionic detergents and proteinase K (50 mM KCl, 10 mM Tris–HCl pH 8.3, 0.1 mg/ml gelatine, 0.45% NP 40, 0.45% Tween 20, 0.3 g/ml proteinase K) and incubated at 60°C for 1 h. Consequently the mix was incubated at 95 ◦ C for 10 min to denature the proteinase K. Finally the DNA was aliquoted into cryo-Vials (NUNK, Germany) and frozen at -70°C till requirement.

IDE genotyping

The *IDE* genotypes were determined using TaqMan assay with the real time PCR reaction using specific primers from TaqMan single nucleotide polymorphism (SNP) Genotyping Assay (Applied Biosystems, Darmstadt, Germany), which uses the 5´ nuclease assay for amplifying and detecting specific SNP alleles in purified genomic DNA samples. C_22272896_10(IDE2, rs4646953), C_27104906_10 (IDE7, rs2251101) and C_12116624_10

(IDE9, rs1887922) assays were used for SNP typing. Analysis of the genotypes was conducted on the iCycler software with allelic discrimination program (Bio-Rad, Munich, Germany).

$A\beta_{1-42}$ plasma level

Plasma levels of amyloid were determined by a double-antibody sandwich enzyme-linked immunosorbent assay method according to the company protocol (Innogenetics NV, Ghent, Belgium). The INNOTEST β-amyloid (1–42) allows the specific and reliable measurement of $A\beta_{42}$ peptides in plasma. The detection range is 5–1000 pg/ml.

Statistical analysis

Prior to association analysis, *IDE* SNPs were tested for Hardy-Weinberg equilibrium (HWE) with a one degree of freedom χ^2-test; no significant departures were detected (all p-values > 0.001). Associations in the different time points were tested with logistic regression when the outcome variable was binary (no/yes), or with linear regression when influence on a continuous outcome was examined. For longitudinal association analysis, generalized estimating equations were used. Genotypic associations were performed using three different models: in the additive model, each individual's risk allele count entered the regression; the dominant model considered the presence of at least one risk allele and in the recessive model, a genotypic risk was only present if the individual was homozygous for the risk allele. The Akaike Information Criterion (AIC) was used to choose the best model. In single SNP analysis the polymorphism's minor allele was assumed to be the risk allele. In haplotype analysis, each haplotype allele was tested against all other alleles; to account for phase uncertainty, each allele's posterior probability was incorporated into the model. Haplotypes were defined with the expectation-maximization algorithm [90]. Associations were considered to be significant at $\alpha=0.05$; due to the limited sample size of this study, the reported nominal p-values were not adjusted for multiple testing. All analyses were performed in R version 2.10.0 using the packages geepack and SimHap (all obtained from http://www.r-project.org).

MANUSCRIPT II

Sample preparation

The brain samples used in this study were supplied by BrainNet Europe. The entire procedure was performed in accordance with the Helsinki Declaration in its latest version and with the Convention of the Council of Europe on Human Rights and Biomedicine. Clinical diagnosis of AD was based on National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria and confirmed by neuropathological findings [91]. Histological samples were obtained from the dorsal and ventral hippocampus and from the prefrontal cortex of post-mortem brains from four patient groups (Table 3): age-matched control patients without dementia, patients with T2DM, AD patients, and patients with AD plus T2DM. In preparation for routine neuropathological examination, the brain was divided midsagittally, and one hemisphere was immersed in 4.5% p-formaldehyde (Fischer GmbH, Saarbruecken Germany) for 3-4 weeks.

Table 3: Demographic data of postmortem brains

	Control	AD	AD+T2DM	T2DM
Age, years **[mean(+/- SEM)]**	69 (+/- 9)	75 (+/-7)	75 (+/- 6)	71 (+/- 6)
Gender (m/w)	4/5	5/5	6/4	4/6
Braak	0-I	IV-VI	III-V	0-I

Abbrevation used: AD= Alzheimer disease; T2DM= type 2 diabetes mellitus; m = male; f = female; **SEM = standard error of mean**

Immunohistochemistry

Paraffin sections (8 µm) of post-mortem brain tissue from the four patient groups were deparaffined with an alcohol dilution series. The slides were then boiled in 10 mM Citratbuffer cointaing 10.51 g citratmonohydrate and 2 g sodium hydroxide pellets in 5l double distillate water (pH 6) for at least 10 min for antigen retrieval. The sections were washed three times in Tris-buffered saline, and non-specific binding was blocked with blocking solution (BS) containing 10% normal goat serum, 2% bovine serum albumin, and 0.01% Triton-X 100 in Tris-buffered saline for 1 h at room temperature before incubation

with the various primary antibodies diluted in BS. The primary antibodies targeted the following proteins: IRß (Santa Cruz, Heidelberg, Germany), diluted 1:200; neuronal specific enolase (NSE; Abcam, Cambridge, UK), diluted 1:300; and PPARγ(p) (Abcam, Cambridge, UK), diluted 1:200 and all antibodies were incubated overnight at 4 °C. The primary antibodies were visualised with antibodies conjugated to Alexa® Fluor-488 (green) and Fluor-555 (red; Invitrogen, Darmstadt, Germany). All secondary antibodies were incubated for 2 h at room temperature in the dark. Finally, the sections were mounted on glass slides and cover slipped under Vectashield (Vector Labs, Eiching, Germany) for fluorescence microscopy.

Automated cell counting

Five images of each brain region from each patient were recorded and analysed with Cell^P (version 2.0; Olympus, Hamburg, Germany). We used Imaging C within Cell^P to use a macro recorder, to define special regions of interest for each investigated protein, and to perform automated cell counting. The total cell number for each image was determined, and we separately obtained counts for the special regions of interest for each image. Automated cell counting helped us maintain the standard error of measurement at the same level for image analyses.

Statistical analysis

Immunohistochemistry images were analysed for the numbers of cells positive for NSE, IRβ, and PPARγ(p) staining in comparison to the total number of cells via analysis of variance (ANOVA) and a post-hoc Scheffé test with a significance level of $p<0.05$. Reported values were calculated in relative to control levels (100%). The statistical program Stat View 5.0 (SAS Institute Inc. Cary, NC, USA) was used for all analyses.

MANUSCRIPT III

Cell culture

SH-SY5Y cells were bought from European Collection of Cell Cultures and grown in buffered Dulbecco`s modified Eagle medium (DMEM/F-12) (Pan Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum and 0.1% gentamycin (50 mg/ml) (Life technologies, Darmstadt, Germany) in a humidified incubator (5% CO_2) at 37°C.

Preparation of soluble and aggregated $A\beta_{42}$

The preparation of the $A\beta_{42}$ peptide was modified according to Dahlgren [92]. 500mg of freeze-dried $A\beta_{42}$ (Millipore, Schwalbach, Germany) was dissolved in 1,1,1,3,3,3 hexafluoroisopropanol (HFIP) to a concentration of 1mM and incubated for one hour at room temperature. Subsequently, the HFIP was removed by using a vacuum centrifuge and the peptide film was dissolved in dry dimethyl sulfoxide (DMSO) to a final concentration of 5mM. For aggregated $A\beta_{42}$ peptides, the solution was diluted with 10mM HCl and stored for 7 days in the incubator at 37°C. The test concentrations of both the soluble and the aggregated $A\beta_{42}$ were finally diluted with normal cell culture medium.

Cell treatment with $A\beta_{42}$

For western blot (WB), enzyme linked immunosorbent assay (ELISA), fluorescent ELISA and quantitative real-time polymerase chain reaction (QRT-PCR), SH-SY5Y cells were treated with varying concentrations of soluble and aggregated $A\beta_{42}$ (see preparation above) (0, 5, 1, 2,5 and 10µM), or purified DMSO or hydrochlorid acid (HCl) as a control, by adding it in the right concentration to the culture medium. The reaction was stopped after 48 h and cells were trypsinized for further investigations.

Cytotoxicity

To determine a possible toxic effect of $A\beta_{42}$ on the cells, the CytoTox-Glo™ test from Promega (Mannheim, Germany) was performed. For testing the cytotoxicity effect, 10.000cells/ml per well were cultivated for 24 hours at 37°C in 5% CO_2 saturation in the incubator, so that the cells could adhere to the bottom of the wells. Afterwards cells were treated with soluble or aggregated $A\beta_{42}$ in a concentration of 0.1, 0.5, 1, 2, 3, 5, 10, 20, 50 and 100 µM. Reaction was measured 48h later.

RNA extraction and QRT-PCR

Total RNA was extracted from SH-SY5Y cells using the RNeasy Plus MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany). QRT-PCR was performed using an iCycler iQ™ Real Time PCR Detection System (Bio-Rad) and the SYBR-Green detection method. The QRT-PCR reaction was optimized according to the manufacturer's instructions. QuantiTech Primer assays for β-actin (*ACTB*; QT00193473), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH,* QT01192646*)*, ribosomal protein L13A (*RPL13A*; QT00089915), peptidylprolyl isomerase A (*PPIA*, QT01866137), β-secretase (*BACE*, QT00084777), glycogen synthase kinase 3α (*GSK3α*, QT00075306), glycogen synthase kinase 3β (*GSK3β*, QT00057134), phosphatidylinisitol-3 kinase (*PI-3K*, QT00035175), insulin degrading enzyme (*IDE*, QT00080773), insulin receptor substrate 1 (*IRS1*, QT00074144) and insulin receptor (*INSR*, QT00082810) were purchased from Qiagen (Hilden, Germany). *ACTB, GAPDH, RPL13A* and *PPIA* were used for normalization according to GeNorm [93]. The amplified transcripts were quantified using the comparative threshold cycle (Ct) analyzed using the BioRad iCycler iQ program.

Western blot

Western blotting was used to detect changes in the protein levels of Insulin receptor (IR) β subunit in SH-SY5Y cells. To determine total protein amount of each sample, Bradford protein assay was conducted (Sigma-Aldrich, Schorndorf, Germany). Samples (50µg of total protein, denaturated with dithiothreitol) were separated on 4-12% Bis-Tris gel and transferred onto nitrocellulose membranes (Life technologies, Darmstadt, Germany). Protein of interest was detected using its specific antibodies: rabbit anti-IRβ (antibody) (1:250, Santa Cruz, Heidelberg, Germany). Blocking the membrane for one hour at room temperature (5% blocking milk in TBS-Tween) and afterwards incubation with primary antibody at 4°C overnight, membranes were incubated with HRP conjugated secondary antibody (1:15.000, Sigma-Aldrich, Schorndorf, Germany) for one hour at room temperature. The IRβ protein immunoreactive bands were detected using the enhanced chemiluminescence (ECL) detection plus system from GE Healthcare (Freiburg, Germany) and quantified using ImageJ software without modifying picture properties (e.g. gain, colour or contrast). For comparison the values of β-actin, as a house keeping protein, were used. The membranes were stripped to remove the attached antibodies. It was followed by one-hour incubation in 5% BSA blocking solution

at room temperature. The murine β-actin antibody (1:5000, Santa Cruz, Heidelberg, Germany) was already linked with HRP and was detected and quantified like IRβ protein.

Enzyme linked immunosorbent assays

The Invitrogen (Darmstadt, Germany) GSK-3β kit is a solid phase sandwich enzyme linked immunosorbent assay (ELISA) and was used to detect and quantify the level of total GSK-3β protein and of GSK-3β protein phosphorylated at serine residue 9. Samples were prepared according to manual. Detected protein amount was normalized with total protein amount of used samples, as detected via Bradford protein assay (Sigma-Aldrich, Schorndorf, Germany).

Enzyme activity

MAO-B enzyme activity was measured by using amplex red MAO assay kit (Life technologies, Darmstadt, Germany). 50µg of total protein (detected via Bradford protein assay) of homogenized SH-SY5Y cells was diluted in reaction buffer and varying concentrations of soluble and aggregated $A\beta_{42}$ (see preparation above) (0.5, 1, 2.5 and 10µM) were added. After 30 minutes incubation at 37 °C, 100µl substrate mix was added and again incubated for 1 hour at 37°C. The endpoint measurement was done in a fluorescence multi plates meter (Novostar) at an excitation of 542 nm and an emission of 590 nm.

Statistical analysis

Statistical analyses of the WB, ELISA and QRT-PCR data were performed using StatView for Windows (SAS Institute Inc., version 5). Statistical tests included analysis of variance (ANOVA) with post-hoc Scheffé Test. Significance was set as $p<0.05$. For a better comparison of the data, we normalized the results and converted the data into percentages. The control group was set as 100%. The results represent at least 3 repeated experiments with around 4 internal repeats.

DISCUSSION

GENERAL DISCUSSION

AD is the most frequent form of dementia in the elderly with an increasing incidence in the aging population in industrialized countries in the next decades. Thus, the investigation of associations between risk factors, possible genetic candidate genes and influences of other disease like T2DM on AD, is of major interest. Several studies have reported a high risk of AD in patients with T2DM [94-98]. Also accumulating evidence supports the hypothesis that AD and T2DM share a common link [79,99,100]; while aging is clearly the strongest risk factor for AD, emerging data suggest that T2DM and dyslipidaemic states can contribute substantially to AD pathogenesis either directly or as cofactors [101]. In addition, the co-morbid hypertension and overweight of T2DM were found to be linked to risk of dementia [58,102-104]. The presented thesis investigates the questions are there common genetic, histopatholigical and/or proteomic features or pathways of AD and T2DM? Summarizing the results of Manuscripts I, II and III leads to the conclusion that even though AD and T2DM are sharing common features like low density of IRβ and higher density of PPARγ (p) (see results Manuscript II) or Aβ_{42} influences insulin signaling proteins (see results Manuscript III), the risk effects were more independent rather than linked, which confirms the term for AD as a Type III Diabetes. In the following chapters, this hypothesis will be further discussed:

Insulin degrading enzyme gene- a link of Alzheimer's disease with type 2 diabetes mellitus?

Search for candidate genes and/or analysis of SNP polymorphisms are getting more and more attention in the scientific community to find a possibility for earlier treatment and better prognosis of symptomatically indistinct illnesses. The presented study laid the main focus on *IDE* as potential link between AD and T2DM (Manuscript I). *IDE* has obtained much attention as a LOAD candidate gene because of its location near to LOAD linkage peaks. The *IDE* gene is located on chromosome 10q23.33, very near a 'suggestive' linkage peak (chromosome 10q24) found several genome-wide linkage studies of LOAD families. reviewed in [105]. Since *IDE* is also well-known to be associated with increased risk for T2DM [69,70,106], the present study aimed at examining the effects of selected common *IDE* polymorphisms on both disease outcomes as well as on the plasma Aβ_{42} level (see Manuscript I). Assuring that the analysed polymorphisms capture the common allelic variation in the up- and downstream region, as well as in the gene body of *IDE,* SNP of IDE2 (upstream), IDE7 (downstream) and IDE9 (gene body) was further investigated. Subjects of the present study

were from the VITA study (see detailed methods Manuscript I). While there was no association detectable of IDE9 with neither AD nor T2DM susceptibility, the presented study could show that allelic variation at opposing ends of *IDE* is associated with different outcomes: the upstream and 5'-untranslated region (UTR) harbors polymorphisms (IDE2) modifying the AD disease risk and the $A\beta_{42}$ plasma level, whereas 3'-UTR and downstream variants (IDE7) trigger T2DM susceptibility. In details the minor C allele of IDE2 had a protective effect with an OR =0.55 for AD and for the minor G allele of IDE7 bears a significant genotypic risk to develop T2DM, with an initial OR of 2.43 (see results Manuscript I). The possible protective effect of IDE2 is not easy to explain. 2009 Zuo and colleagues [66] could demonstrate that variants of polymorphism inside the *IDE* upstream region influences the expression of *IDE* in an enhanced but also in an inhibited manner. In addition previous studies of *IDE* expression in AD brain tissue have produced inconsistent results (reviewed in [107]). Although increased intracellular and neuronal immunostaining have been reported in AD brains, decreased levels of a carboxyl-terminal fragment of IDE were detected in cytosolic fractions of AD brains by Western blot analysis. Additionally IDE protein levels measured by enzyme linked immunosorbent assay were reported to be increased in the cortical microvessels of AD patients In contrast, decreased IDE activity seems to be link with AD [52]. The detected protective effect of IDE2 for AD could either result in a lower or in a higher IDE expression/activity prompting further investigations.

The reported association of the downstream variant IDE7 with T2DM might be an indirect signal that extends from the *IDE* 3'-UTR over LD (Manuscript I). T2DM is characterised as insulin resistance or insulin receptor insensitivities. Insulin resistance begins years before the onset of T2DM [108]. Thus the presumed risk allele may attenuate translation or reduce the stability of the *IDE* mRNA, thus leading to less IDE activity and in a consequence less degrading of insulin, which can lead to a hyperinsulinemia and so explaining the increased T2DM susceptibility. Based on these assumptions, the presented study delineate the model that polymorphisms at opposing ends of the *IDE* gene lead to expression changes with consequences on susceptibility to different diseases: promoter variation increases *IDE* expression and protects from AD, while 3'-UTR variation decreases *IDE* expression and increases T2DM risk. To concluded the polymorphisms of *IDE* seem to have opposing effects on *IDE* expression, a relevant endophenotype with disorder-specific effects on AD and T2DM susceptibility but not a common effect on both diseases. For more details see Manuscript I.

Common histopathological features of Alzheimer's disease and type 2 diabetes mellitus

Identifying potential common links between AD and T2DM makes it necessary not only to look on genetically influences of humans, it is also important to investigate directly human brain tissue for similar and different alterations in T2DM and AD. Because of the neurodegenerative decline in AD patients and also because of insulin is known to be synthesized in the brain as well as it is very important for cognition and memory [109] the brain should be in a special focus for the investigations of common links. For this issue the presented thesis analyzed 10 post-mortem brain tissues of four different groups: AD, AD combined with T2DM, T2DM and age-matched controls (see detailed methods Manuscript II). In all investigated patient groups significantly lower density of IRβ in the hippocampus region could be detected and additionally patient groups possessed significantly more PPARγ(p) positive cells than the control group in the investigated brain regions (Manuscript II). The particular study was focused especially on the phosphorylated form of PPARγ, which is the inactive variant of this protein [110] and which seems to play an important role in the pathophysiology of T2DM and maybe also in AD. PPARγ agonists improve insulin sensitivity by decreasing the level of circulating insulin, increasing insulin-mediated glucose uptake, and enhancing insulin action in the brain [111] . It was demonstrated that six months treatment with rosiglitazone, a typical T2DM treatment and a PPARγ agonist, preserved cognitive function for patients with AD and amnestic mild cognitive impairment compared with a placebo-treated group [83]. Several studies have examined potential mechanisms by which PPARγ agonists may ameliorate AD pathogenesis and progression (e.g. diminish proinflammatory gene expression and cytokine release [112], enhancing of antioxidant enzymes [113], improve to restore glucose uptake in the brain [114]) and therefore it was not surprising to observe higher numbers of the inactive and not protective form PPARγ(p) positive cells in the brains of all patient groups compared to the control group. However, the presented study could not detect differences between the different patient groups (all groups were almost heavily affected, see results Manuscript II) suggesting that PPARγ(p) expression is equally enhanced in AD and T2DM and this fact seems to give a hint why PPARγ agonists having positive effects in AD patients as well as in T2DM patients.

An interesting result of the study was that not only AD patients had a lower IRβ density in the brain, also the T2DM patient showed a lower density in the hippocampus. For AD it was already described that post-mortem cases of LOAD are associated with strikingly reduced levels of insulin, IGF-1 polypeptide, and related receptor genes in the whole brain [75] and so a

lower IRβ density in brain regions of AD patients fits into this circumstance. But it still does not solve the question, why should also T2DM patients have lower IRβ level in the hippocampus? T2DM is defined via insulin resistance, which is characterized by reduced responsiveness of IRs and decreased downstream signaling for the purpose of insulin stimulation. To compensate for these dysfunctions, the islet β-cells of the pancreas secrete more insulin, thereby creating a state of hyperinsulinemia another hallmark of T2DM [115]. These facts are very well investigated in the periphery, but up to date no research group investigated the brains of T2DM patients with a special focus on IR density like in this study. For answering the question of lower IR density the insulin pathway and the link to the IR expression has to be further enlighten. As already mention in figure 3 (see Introduction) insulin activates the transmembrane IR leading to the activation of intracellular IRS. Active IRS leads to phosphorylation of PI3K, which activates AKT. Active AKT not only inhibits GSK3 it also inhibits forkhead box O (FOXO), which is a transcription factor that induces IR expression [116]. In case of hyperinsulinemia, an overproduction of insulin exists in the body and so a negative feedback loop starts, which can lead to a lower density of IR (figure 5).

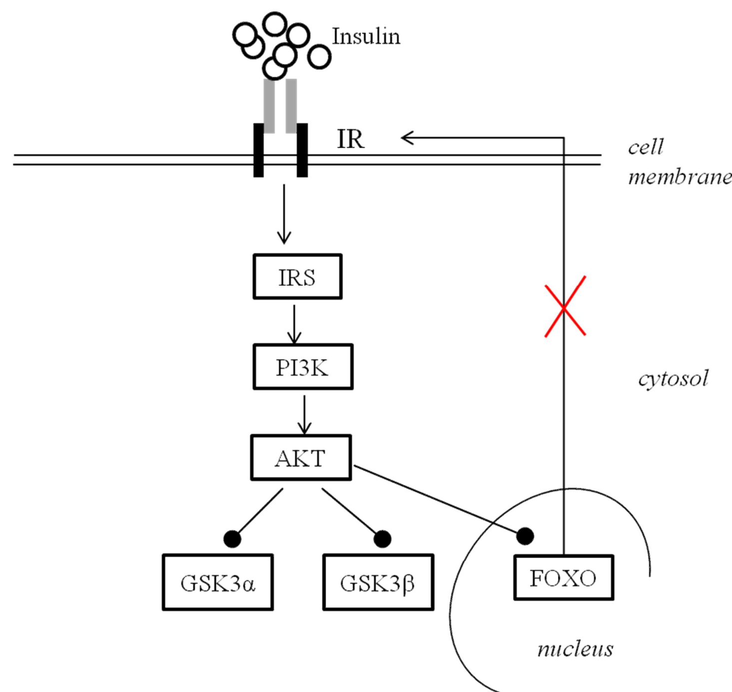


Figure 5: negative feedback loop of insulin and insulin receptor expression. Insulin activates the transmembrane insulin receptor (IR) leading to the activation of intracellular insulin receptor substrate (IRS). Active IRS leads to phosphorylation of phosphoinositide-3 kinase (PI3K), which activates protein kinase B (AKT). Once active, AKT leads to the phosphorylation and consequently to the inactivation transcription factor forkhead box O (FOXO). Inhibited FOXO leads to a lower or inhibited IR gene expression. → = activation; • = inhibition; x= inactive gene expression.

The presented result (Manuscripts II) can be explained via this negative feedback loop of insulin and IR. Unfortunately the study was unable to confirm the expectation that patients with AD plus T2DM had significantly fewer IRβ positive cells compared with the single disease groups. Thus, it seems that IRβ loss in the brain is a restricted phenomenon, and cannot decrease past a certain level.

In conclusion, T2DM and AD may not be directly linked, but may share common histological features including lower numbers of IRβ positive cells in the hippocampus and higher numbers of PPARγ(p) positive cells in the prefrontal cortex and the hippocampus, which affirms that AD seems to be a type III diabetes. For more details see Manuscript II.

Aggregation form of amyloid β_{42} influences gene/ protein expression

Although more and more factors are found to be involved in AD as described in the previous chapters, excessive $A\beta_{42}$ deposition is still considered to play a major role in AD. While post-mortem studies are necessary for investigation of changes within the brain of AD patients, it is difficult to distinguish whether changes in gene-/protein expression were elicited directly by $A\beta_{42}$ signaling or were a consequence of events taking place at later stage of the disease. Thus the presented thesis also used the neuroblastoma cell line SH-SY5Y (see detailed methods Manuscript III) to determine directly whether neurons exposed to $A\beta_{42}$ peptides undergo pathological changes characteristic of AD brain and which influences do they have on genes and proteins involved in the insulin signaling pathway and in the APP pathway. Generally it is accepted that the oligomeric form of $A\beta_{42}$ causes synapse dysfunction, including Tau hyperphosphorylation, calcium dysregulation and oxidative stress blockade of fast axonal transport altered turnover of neuronal receptors involved in synaptic plasticity, and synapse loss, the molecular and cellular mechanisms underlying their toxicity are still poorly understood and need further clarification [117]. The presented study investigated the effects of both soluble oligomeric and insoluble aggregated $A\beta_{42}$ peptides on gene expression, protein level and enzyme activity *in vitro* to determine differences of the physical shape of the peptides. In particular, it was not possible to detect any significant influences of the different forms of $A\beta_{42}$ peptides on *IDE, IRS1, BACE*, *GSK3α* or *PI3K* gene expression, but a strong influence of the soluble form of $A\beta_{42}$ peptides on *IR* and *GSK3β* gene expression could be observed as well as on protein level (see results Manuscript III). Although it couldn't be shown an influence of $A\beta_{42}$ on *GSK3α* gene expression, the result of increased GSK3β gene/protein expression and also detected enhanced activity points to the fact, that GSK3β

might be a crucial element in the $A\beta_{42}$-triggered molecular cascade leading to potential neurodegeneration. The GSK3β activity was implicated in tau phosphorylation, APP processing, $A\beta_{42}$ production and neurodegeneration, which are all features of AD [48,118]. Accordingly, over-expression of GSK3β in mice prevents the induction of long term potentiation (LTP) [119] and causes a decrease in spatial learning [120]. Inhibitors of GSK3β have also been shown to block long-term depression (LTD) and GSK3β activity is enhanced during LTD [121]. Thus, it would appear that GSK3β is critical for the induction of memory formation, switching off LTD and allowing LTP to occur. The observed effect of $A\beta_{42}$ oligomers on GSK3β might be mediated by interaction with different receptors, but mainly with the interaction of IR. The signal transduction by neuronal IR is strikingly sensitive to disruption by soluble $A\beta_{42}$ oligomers [122] and so the presented data indicate the fact that this relationship has the potential to generate an insidious pathogenic loop. It could be observed a significant reduction of IR gene and protein expression after SH-SY5Y treatment with soluble $A\beta_{42}$ oligomers compared to untreated cells. Consistent with the actual finding reported here, it could be already demonstrated that AD patients have less IR in hippocampus and in prefrontal cortex (see Manuscript II). Not only an impairment of IR leads to an activation of GSK3β as explained before, also OS can enhance the activity of this kinase [123]. OS can result from the formation of H_2O_2 as a by-product of metabolism of aminergic neurotransmitters through MAO-B. It has been demonstrated that inhibitors of MAO-B, such as l-deprenyl and, more recently, rasagiline, are effective in the management of early symptoms of Parkinson's disease in the clinic and in animal models as well as in patients with mild AD [124]. Furthermore ROS have been found in the vicinity of amyloid plaques [125] and increased MAO-B activity was found especially in platelets of AD patients [126]. Therefore the presented results of enhanced MAO-B activity following treatment with aggregated while not with soluble $A\beta_{42}$ peptides, might indicate the involvement of the fibrillar structures of $A\beta_{42}$ in the OS cascade known to play an important role in AD [127,128]. Anyhow the presented thesis is the first report indicating the direct influence of soluble or aggregated peptides on MAO-B activity *in vitro* and the presented data affirm that there has to be a direct neurobiochemical link between MAO-B and $A\beta_{42}$. OS is not only involved in AD, it seems to contribute the development of microvascular and cardiovascular diseases in patients with T2DM [129], especially excessive ROS impair insulin synthesis and activate β-cell apoptotic signaling pathways. Additionally ROS may lead to inhibition of signaling at the level of IRS phosphorylation and insulin resistance [130]. A recent study of Nunes and colleagues investigated the activity of MAO-B in T2DM patients

without finding any significant changes [131], which leads to the conclusion that OS is involved in the pathogenesis of T2DM but independent of MAO-B activity.

In conclusion, our results suggest that $A\beta_{42}$ oligomers seem to trigger more cellular mechanisms linked to AD than the insoluble aggregated $A\beta_{42}$ peptides.

OUTLOOK

Based on current literature and the results presented in this thesis, there is an association between impaired cerebral insulin signaling pathway and AD, but it is still needs to be discerned whether it is a consequence, a result, or an epiphenomenon. Concerning of the existing investigation of *IDE* and the potentially protective effect on AD, a further step should be to analyze the influence of the SNPs on IDE enzyme activity within the brain. Sampling of post-mortem tissues of participant originating from the VITA cohort has already started, leading to an outstanding resource for AD research. Finding differences of IDE activity in AD patients compared to controls can help to develop potential new therapeutic targets, independent of BACE inhibition as main focus of the recent drug development. A further promising direction will be to study the direct influences of $A\beta_{42}$ peptides on human neurons, especially of soluble peptides on insulin signaling pathways as well as synaptic genes/proteins, or those involved in neuronal survival. Taken into account that during the past 100 years of research in AD neither an effective causative therapy nor preventative strategy has been developed, understanding of etiology and molecular pathogenesis of AD appears as natural prerequisite for this. Considering the increased incidence of AD in the next few decades, investigating molecular pathogenesis as a basis for developing cure and prevention of AD will be one of the main medical challenges of our century.

ACKNOWLEDGEMENT

Zu allererst möchte ich mich bei **PD Dr. Edna Grünblatt** bedanken, da sie mich immer mit vollem Tatendrang unterstützt hat; ich durch sie immer einen Experten an meiner Seite hatte; sie mich immer auf Augenhöhe gesehen hat und da sie mich immer als vollwertige und qualifizierte Wissenschaftlerin betrachtet hat. Zusätzlich möchte ich hier auch direkt **Prof. Dr. Peter Riederer** danken, da er mir immer mit Rat und Tat zur Seite gestand hat und dass er es geschafft haben mich für die Alzheimer Forschung so zu begeistern. Er war immer ein ganz entscheidender Ansprechpartner für mich und hat mit seiner Gastfreundschaft für ein tolles Arbeitsklima gesorgt. Vielen Dank für alles!

Prof. Dr. Erich Buchner möchte ich danken, da er die biologische Betreuung der Arbeit übernommen hat und als Neurobiologe sich auch bestens mit dem gewählten Forschungsgebiet auskennt und somit auch ein sehr wichtiger Bestandteil für meine Dissertation ist.
Ein recht herzliches Dankeschön dafür.

Von ganzem Herzen möchte ich meiner Familie danken. Ohne meine geliebten Eltern **Rosi** und **Dieter Bartl** hätte ich schon längst kapituliert und ohne ihren Glauben an mich, hätte ich niemals so viel erreicht in meinem Leben. Ich bin wahnsinnig stolz, dass ich so tolle Eltern habe auf die ich IMMER zählen kann und die mich bedingungslos lieben. Ihr seid einfach so ganz wunderbare Menschen und ich kann euch nicht genug für alles danken. Ich kann mir nichts Besseres wünschen als eure Tochter zu sein!
Meiner bezaubernden Schwester **Sabrina** möchte ich auch ganz besonders danken, denn es ist sicherlich nicht leicht mich als Schwester zu haben, aber sie hat mir immer beigestanden egal was passiert ist und hat so manche Laune von mir stumm ertragen. Ich hab dich lieb!

Ein großer Dank geht natürlich auch an die gesamte Laborcrew der Psychiatrie. Mei, ohne euch hätte ich oft mehr geweint als gelacht während der alltäglichen Laborarbeit, aber ihr habt es immer geschafft mich aufzubauen, mich zum Lachen zu bringen und mit mir so manches grandioses Fest gefeiert. Ihr werdet mir alles sehr fehlen, aber besonders werde ich **Lise**, **Joyce**, **Julia**, **Sissi**, **Terri, Lena, Gabi** und **Carola** vermissen. Danke, dass ich so tolle Leute um mich herum hatte, die mir einfach sehr ans Herz gewachsen sind und denen ich hin und wieder ein Ohr abkauen durfte. Ich werde euch wirklich schrecklich vermissen! Hier müssen auch meine ganzen Studenten, die einen wichtigen Beitrag zu dieser Dissertation geleistet

haben, erwähnt werden: **Andrea, Svenja, Anne, Jann** und **Milena** vielen Dank für eure Unterstützung und Mithilfe!

Ein sehr großer DANK geht an meine allerbesten Freunde **Charlotte**, **Mela** und **Caro**. Ich kann die ganzen Stunden, die ihr mir zugehört habt, mich unterstütz habt und für mich da ward, nicht mehr zählen und kein Gold der Welt hat euren Wert; ihr seid schlicht und ergreifend unbezahlbar. Ihr gehört definitiv zu den wichtigsten Personen in meinem Leben und ich weiß, dass das immer so sein wird!! Ihr seid einfach einzigartig und ich kann nicht beschreiben wie toll es ist euch als Freunde zu haben und wie unendlich dankbar ich dafür bin!!!!

Nicht zu vergessen sind hier auch **Manuela, Katja, Phil, Ibo, Andi, Ingrid** und **Mimi,** die mit mir geflucht, getrunken, Stunden telefoniert, gelacht, geweint, so manches philosophische Gespräch geführt und so manchen Sturz erlebt haben. Ihr seid das Salz in meiner Suppe und jeder einzelne von euch ist ein ganz besonderer Mensch, auch wenn ich dies vielleicht nicht immer so direkt zeigen konnte. ABER ihr sollt wissen wie wichtig ihr für mich seid und immer bleiben werdet!

Und zum Schluss geht mein Dank auch noch an den Helfer im Hintergrund…ich danke dir von meinem ganzen sizilianischen Herzen und nicht nur für deine Hilfe bei dieser Dissertation, aber ich hoffe das weißt du bereits. Gracie!!

REFERENCES

1. Alzheimer A, Stelzmann RA, Schnitzlein HN, Murtagh FR. An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde". Clin Anat. 1995;8:429-431.
2. Mirra SS, Heyman A, McKeel D, et al. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology. 1991;41:479-486.
3. Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. 1991;82:239-259.
4. Wilson RS, Schneider JA, Arnold SE, Tang Y, Boyle PA, Bennett DA. Olfactory identification and incidence of mild cognitive impairment in older age. Arch Gen Psychiatry. 2007;64:802-808.
5. Isik AT. Late onset Alzheimer's disease in older people. Clin Interv Aging;5:307-311.
6. Molsa PK, Marttila RJ, Rinne UK. Long-term survival and predictors of mortality in Alzheimer's disease and multi-infarct dementia. Acta Neurol Scand. 1995;91:159-164.
7. Bi X. Alzheimer disease: update on basic mechanisms. J Am Osteopath Assoc. 2010;110:S3-9.
8. Bird TD. Genetic aspects of Alzheimer disease. Genet Med. 2008;10:231-239.
9. Bekris LM, Yu CE, Bird TD, Tsuang DW. Genetics of Alzheimer disease. J Geriatr Psychiatry Neurol. 2010;23:213-227.
10. Querfurth HW, LaFerla FM. Alzheimer's disease. N Engl J Med. 2010;362:329-344.
11. Isik AT. Late onset Alzheimer's disease in older people. Clin Interv Aging. 2010;5:307-311.
12. De Strooper B. Proteases and proteolysis in Alzheimer disease: a multifactorial view on the disease process. Physiol Rev. 2010;90:465-494.
13. Turner PR, O'Connor K, Tate WP, Abraham WC. Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. Prog Neurobiol. 2003;70:1-32.
14. Duce JA, Tsatsanis A, Cater MA, et al. Iron-export ferroxidase activity of beta-amyloid precursor protein is inhibited by zinc in Alzheimer's disease. Cell. 2010;142:857-867.
15. Sanchez-Alavez M, Chan SL, Mattson MP, Criado JR. Electrophysiological and cerebrovascular effects of the alpha-secretase-derived form of amyloid precursor protein in young and middle-aged rats. Brain Res. 2007;1131:112-117.
16. Leissring MA, Murphy MP, Mead TR, et al. A physiologic signaling role for the gamma -secretase-derived intracellular fragment of APP. Proc Natl Acad Sci U S A. 2002;99:4697-4702.
17. Nunan J, Small DH. Regulation of APP cleavage by alpha-, beta- and gamma-secretases. FEBS Lett. 2000;483:6-10.
18. Crouch PJ, Harding SM, White AR, Camakaris J, Bush AI, Masters CL. Mechanisms of A beta mediated neurodegeneration in Alzheimer's disease. Int J Biochem Cell Biol. 2008;40:181-198.
19. Gotz J, Eckert A, Matamales M, Ittner LM, Liu X. Modes of Abeta toxicity in Alzheimer's disease. Cell Mol Life Sci. 2011;68:3359-3375.
20. Parihar MS, Brewer GJ. Amyloid-beta as a modulator of synaptic plasticity. J Alzheimers Dis. 2010;22:741-763.
21. Glabe CG. Structural classification of toxic amyloid oligomers. J Biol Chem. 2008;283:29639-29643.
22. Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol. 2007;8:101-112.
23. De Felice FG, Wu D, Lambert MP, et al. Alzheimer's disease-type neuronal tau hyperphosphorylation induced by A beta oligomers. Neurobiol Aging. 2008;29:1334-1347.
24. Klyubin I, Betts V, Welzel AT, et al. Amyloid beta protein dimer-containing human CSF disrupts synaptic plasticity: prevention by systemic passive immunization. J Neurosci. 2008;28:4231-4237.
25. Cleary JP, Walsh DM, Hofmeister JJ, et al. Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. Nat Neurosci. 2005;8:79-84.

26. Walsh DM, Klyubin I, Shankar GM, et al. The role of cell-derived oligomers of Abeta in Alzheimer's disease and avenues for therapeutic intervention. Biochem Soc Trans. 2005;33:1087-1090.
27. Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL. Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J Neurosci. 2007;27:2866-2875.
28. Alberdi E, Sanchez-Gomez MV, Cavaliere F, et al. Amyloid beta oligomers induce Ca2+ dysregulation and neuronal death through activation of ionotropic glutamate receptors. Cell Calcium. 2010;47:264-272.
29. Tamagno E, Bardini P, Guglielmotto M, Danni O, Tabaton M. The various aggregation states of beta-amyloid 1-42 mediate different effects on oxidative stress, neurodegeneration, and BACE-1 expression. Free Radic Biol Med. 2006;41:202-212.
30. Tang BL. Neuronal protein trafficking associated with Alzheimer disease: from APP and BACE1 to glutamate receptors. Cell Adh Migr. 2009;3:118-128.
31. Verdier Y, Zarandi M, Penke B. Amyloid beta-peptide interactions with neuronal and glial cell plasma membrane: binding sites and implications for Alzheimer's disease. J Pept Sci. 2004;10:229-248.
32. Diaz JC, Simakova O, Jacobson KA, Arispe N, Pollard HB. Small molecule blockers of the Alzheimer Abeta calcium channel potently protect neurons from Abeta cytotoxicity. Proc Natl Acad Sci U S A. 2009;106:3348-3353.
33. Bores GM, Smith CP, Wirtz-Brugger F, Giovanni A. Amyloid beta-peptides inhibit Na+/K+-ATPase: tissue slices versus primary cultures. Brain Res Bull. 1998;46:423-427.
34. Riederer P, Danielczyk W, Grunblatt E. Monoamine oxidase-B inhibition in Alzheimer's disease. Neurotoxicology. 2004;25:271-277.
35. Grunblatt E, Bartl J, Riederer P. The link between iron, metabolic syndrome, and Alzheimer's disease. J Neural Transm. 2010;118:371-379.
36. Pratico D. Oxidative stress hypothesis in Alzheimer's disease: a reappraisal. Trends Pharmacol Sci. 2008;29:609-615.
37. Tabaton M, Zhu X, Perry G, Smith MA, Giliberto L. Signaling effect of amyloid-beta(42) on the processing of AbetaPP. Exp Neurol. 2009;221:18-25.
38. Zhao WQ, Lacor PN, Chen H, et al. Insulin receptor dysfunction impairs cellular clearance of neurotoxic oligomeric a{beta}. J Biol Chem. 2009;284:18742-18753.
39. Townsend M, Mehta T, Selkoe DJ. Soluble Abeta inhibits specific signal transduction cascades common to the insulin receptor pathway. J Biol Chem. 2007;282:33305-33312.
40. Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol. 2001;11:297-305.
41. Zhang Y, Hong Y, Bounhar Y, et al. p75 neurotrophin receptor protects primary cultures of human neurons against extracellular amyloid beta peptide cytotoxicity. J Neurosci. 2003;23:7385-7394.
42. Lesne S, Gabriel C, Nelson DA, et al. Akt-dependent expression of NAIP-1 protects neurons against amyloid-{beta} toxicity. J Biol Chem. 2005;280:24941-24947.
43. Jolivalt CG, Lee CA, Beiswenger KK, et al. Defective insulin signaling pathway and increased glycogen synthase kinase-3 activity in the brain of diabetic mice: parallels with Alzheimer's disease and correction by insulin. J Neurosci Res. 2008;86:3265-3274.
44. Welsh GI, Proud CG. Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. Biochem J. 1993;294 (Pt 3):625-629.
45. Troussard AA, Tan C, Yoganathan TN, Dedhar S. Cell-extracellular matrix interactions stimulate the AP-1 transcription factor in an integrin-linked kinase- and glycogen synthase kinase 3-dependent manner. Mol Cell Biol. 1999;19:7420-7427.
46. Turenne GA, Price BD. Glycogen synthase kinase3 beta phosphorylates serine 33 of p53 and activates p53's transcriptional activity. BMC Cell Biol. 2001;2:12.
47. Anderton BH, Betts J, Blackstock WP, et al. Sites of phosphorylation in tau and factors affecting their regulation. Biochem Soc Symp. 2001:73-80.

48. Hooper C, Killick R, Lovestone S. The GSK3 hypothesis of Alzheimer's disease. J Neurochem. 2008;104:1433-1439.
49. Mukherjee A, Song E, Kihiko-Ehmann M, et al. Insulysin hydrolyzes amyloid beta peptides to products that are neither neurotoxic nor deposit on amyloid plaques. J Neurosci. 2000;20:8745-8749.
50. Kurochkin IV, Goto S. Alzheimer's beta-amyloid peptide specifically interacts with and is degraded by insulin degrading enzyme. FEBS Lett. 1994;345:33-37.
51. McDermott JR, Gibson AM. Degradation of Alzheimer's beta-amyloid protein by human and rat brain peptidases: involvement of insulin-degrading enzyme. Neurochem Res. 1997;22:49-56.
52. Perez A, Morelli L, Cresto JC, Castano EM. Degradation of soluble amyloid beta-peptides 1-40, 1-42, and the Dutch variant 1-40Q by insulin degrading enzyme from Alzheimer disease and control brains. Neurochem Res. 2000;25:247-255.
53. Qiu WQ, Ye Z, Kholodenko D, Seubert P, Selkoe DJ. Degradation of amyloid beta-protein by a metalloprotease secreted by microglia and other neural and non-neural cells. J Biol Chem. 1997;272:6641-6646.
54. Qiu WQ, Walsh DM, Ye Z, et al. Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. J Biol Chem. 1998;273:32730-32738.
55. de la Monte SM, Wands JR. Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. J Alzheimers Dis. 2005;7:45-61.
56. Biessels GJ, Kappelle LJ. Increased risk of Alzheimer's disease in Type II diabetes: insulin resistance of the brain or insulin-induced amyloid pathology? Biochem Soc Trans. 2005;33:1041-1044.
57. Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM. Diabetes mellitus and the risk of dementia: The Rotterdam Study. Neurology. 1999;53:1937-1942.
58. Luchsinger JA, Gustafson DR. Adiposity, type 2 diabetes, and Alzheimer's disease. J Alzheimers Dis. 2009;16:693-704.
59. Arnaiz E, Jelic V, Almkvist O, et al. Impaired cerebral glucose metabolism and cognitive functioning predict deterioration in mild cognitive impairment. Neuroreport. 2001;12:851-855.
60. Watson GS, Craft S. Modulation of memory by insulin and glucose: neuropsychological observations in Alzheimer's disease. Eur J Pharmacol. 2004;490:97-113.
61. Kroner Z. The relationship between Alzheimer's disease and diabetes: Type 3 diabetes? Altern Med Rev. 2009;14:373-379.
62. Zhao L, Teter B, Morihara T, et al. Insulin-degrading enzyme as a downstream target of insulin receptor signaling cascade: implications for Alzheimer's disease intervention. J Neurosci. 2004;24:11120-11126.
63. Myers A, Holmans P, Marshall H, et al. Susceptibility locus for Alzheimer's disease on chromosome 10. Science. 2000;290:2304-2305.
64. Li YJ, Scott WK, Hedges DJ, et al. Age at onset in two common neurodegenerative diseases is genetically controlled. Am J Hum Genet. 2002;70:985-993.
65. Ertekin-Taner N, Graff-Radford N, Younkin LH, et al. Linkage of plasma Abeta42 to a quantitative locus on chromosome 10 in late-onset Alzheimer's disease pedigrees. Science. 2000;290:2303-2304.
66. Zuo X, Jia J. Promoter polymorphisms which modulate insulin degrading enzyme expression may increase susceptibility to Alzheimer's disease. Brain Res. 2009;1249:1-8.
67. Ghosh S, Watanabe RM, Valle TT, et al. The Finland-United States investigation of non-insulin-dependent diabetes mellitus genetics (FUSION) study. I. An autosomal genome scan for genes that predispose to type 2 diabetes. Am J Hum Genet. 2000;67:1174-1185.
68. Meigs JB, Panhuysen CI, Myers RH, Wilson PW, Cupples LA. A genome-wide scan for loci linked to plasma levels of glucose and HbA(1c) in a community-based sample of Caucasian pedigrees: The Framingham Offspring Study. Diabetes. 2002;51:833-840.
69. Duggirala R, Blangero J, Almasy L, et al. Linkage of type 2 diabetes mellitus and of age at onset to a genetic location on chromosome 10q in Mexican Americans. Am J Hum Genet. 1999;64:1127-1140.

70. Vionnet N, Hani EH, Dupont S, et al. Genomewide search for type 2 diabetes-susceptibility genes in French whites: evidence for a novel susceptibility locus for early-onset diabetes on chromosome 3q27-qter and independent replication of a type 2-diabetes locus on chromosome 1q21-q24. Am J Hum Genet. 2000;67:1470-1480.
71. Carro E, Trejo JL, Gerber A, et al. Therapeutic actions of insulin-like growth factor I on APP/PS2 mice with severe brain amyloidosis. Neurobiol Aging. 2006;27:1250-1257.
72. Deane R, Wu Z, Zlokovic BV. RAGE (yin) versus LRP (yang) balance regulates alzheimer amyloid beta-peptide clearance through transport across the blood-brain barrier. Stroke. 2004;35:2628-2631.
73. Hoyer S. The brain insulin signal transduction system and sporadic (type II) Alzheimer disease: an update. J Neural Transm. 2002;109:341-360.
74. Hoyer S. The aging brain. Changes in the neuronal insulin/insulin receptor signal transduction cascade trigger late-onset sporadic Alzheimer disease (SAD). A mini-review. J Neural Transm. 2002;109:991-1002.
75. Steen E, Terry BM, Rivera EJ, et al. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? J Alzheimers Dis. 2005;7:63-80.
76. Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S. Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. J Neurochem. 2007;101:757-770.
77. Nitsch R, Hoyer S. Local action of the diabetogenic drug, streptozotocin, on glucose and energy metabolism in rat brain cortex. Neurosci Lett. 1991;128:199-202.
78. Duelli R, Schrock H, Kuschinsky W, Hoyer S. Intracerebroventricular injection of streptozotocin induces discrete local changes in cerebral glucose utilization in rats. Int J Dev Neurosci. 1994;12:737-743.
79. de la Monte SM. Insulin resistance and Alzheimer's disease. BMB Rep. 2009;42:475-481.
80. de la Monte SM. Contributions of brain insulin resistance and deficiency in amyloid-related neurodegeneration in Alzheimer's disease. Drugs. 2012;72:49-66.
81. Kersten S, Desvergne B, Wahli W. Roles of PPARs in health and disease. Nature. 2000;405:421-424.
82. Landreth G, Jiang Q, Mandrekar S, Heneka M. PPARgamma agonists as therapeutics for the treatment of Alzheimer's disease. Neurotherapeutics. 2008;5:481-489.
83. Watson GS, Cholerton BA, Reger MA, et al. Preserved cognition in patients with early Alzheimer disease and amnestic mild cognitive impairment during treatment with rosiglitazone: a preliminary study. Am J Geriatr Psychiatry. 2005;13:950-958.
84. Sastre M, Dewachter I, Landreth GE, et al. Nonsteroidal anti-inflammatory drugs and peroxisome proliferator-activated receptor-gamma agonists modulate immunostimulated processing of amyloid precursor protein through regulation of beta-secretase. J Neurosci. 2003;23:9796-9804.
85. Rossner S, Sastre M, Bourne K, Lichtenthaler SF. Transcriptional and translational regulation of BACE1 expression--implications for Alzheimer's disease. Prog Neurobiol. 2006;79:95-111.
86. Sastre M, Walter J, Gentleman SM. Interactions between APP secretases and inflammatory mediators. J Neuroinflammation. 2008;5:25.
87. d'Abramo C, Massone S, Zingg JM, et al. Role of peroxisome proliferator-activated receptor gamma in amyloid precursor protein processing and amyloid beta-mediated cell death. Biochem J. 2005;391:693-698.
88. Fischer P, Jungwirth S, Krampla W, et al. Vienna Transdanube Aging "VITA": study design, recruitment strategies and level of participation. J Neural Transm Suppl. 2002:105-116.
89. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology. 1984;34:939-944.
90. Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. Mol Biol Evol. 1995;12:921-927.

91. Braak H, Braak E. Demonstration of amyloid deposits and neurofibrillary changes in whole brain sections. Brain Pathol. 1991;1:213-216.
92. Dahlgren KN, Manelli AM, Stine WB, Jr., Baker LK, Krafft GA, LaDu MJ. Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. J Biol Chem. 2002;277:32046-32053.
93. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3:RESEARCH0034.
94. Ott A, Stolk RP, Hofman A, van Harskamp F, Grobbee DE, Breteler MM. Association of diabetes mellitus and dementia: the Rotterdam Study. Diabetologia. 1996;39:1392-1397.
95. Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM. Diabetes mellitus and the risk of dementia: The Rotterdam Study. Neurology. 1999;53:1937-1942.
96. Leibson CL, Rocca WA, Hanson VA, et al. Risk of dementia among persons with diabetes mellitus: a population-based cohort study. Am J Epidemiol. 1997;145:301-308.
97. Hassing LB, Grant MD, Hofer SM, et al. Type 2 diabetes mellitus contributes to cognitive decline in old age: a longitudinal population-based study. J Int Neuropsychol Soc. 2004;10:599-607.
98. Whitmer RA. Type 2 diabetes and risk of cognitive impairment and dementia. Curr Neurol Neurosci Rep. 2007;7:373-380.
99. J SR-F, Sa-Roriz TM, Rosset I, et al. (Pre)diabetes, brain aging, and cognition. Biochim Biophys Acta. 2009;1792:432-443.
100. Sims-Robinson C, Kim B, Rosko A, Feldman EL. How does diabetes accelerate Alzheimer disease pathology? Nat Rev Neurol. 2010;6:551-559.
101. Qiu C, De Ronchi D, Fratiglioni L. The epidemiology of the dementias: an update. Curr Opin Psychiatry. 2007;20:380-385.
102. Hassing LB, Dahl AK, Thorvaldsson V, et al. Overweight in midlife and risk of dementia: a 40-year follow-up study. Int J Obes (Lond). 2009;33:893-898.
103. Hassing LB, Hofer SM, Nilsson SE, et al. Comorbid type 2 diabetes mellitus and hypertension exacerbates cognitive decline: evidence from a longitudinal study. Age Ageing. 2004;33:355-361.
104. Whitmer RA, Gustafson DR, Barrett-Connor E, Haan MN, Gunderson EP, Yaffe K. Central obesity and increased risk of dementia more than three decades later. Neurology. 2008;71:1057-1064.
105. Bertram L, McQueen MB, Mullin K, Blacker D, Tanzi RE. Systematic meta-analyses of Alzheimer disease genetic association studies: the AlzGene database. Nat Genet. 2007;39:17-23.
106. Karamohamed S, Demissie S, Volcjak J, et al. Polymorphisms in the insulin-degrading enzyme gene are associated with type 2 diabetes in men from the NHLBI Framingham Heart Study. Diabetes. 2003;52:1562-1567.
107. Kim M, Hersh LB, Leissring MA, et al. Decreased catalytic activity of the insulin-degrading enzyme in chromosome 10-linked Alzheimer disease families. J Biol Chem. 2007;282:7825-7832.
108. Aoki TJ, White RD. Initiating insulin in patients with type 2 diabetes. J Fam Pract. 2007;56:S12-20.
109. Strachan MW, Deary IJ, Ewing FM, Frier BM. Is type II diabetes associated with an increased risk of cognitive dysfunction? A critical review of published studies. Diabetes Care. 1997;20:438-445.
110. Adams M, Reginato MJ, Shao D, Lazar MA, Chatterjee VK. Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. J Biol Chem. 1997;272:5128-5132.
111. Sato T, Hanyu H, Hirao K, Kanetaka H, Sakurai H, Iwamoto T. Efficacy of PPAR-gamma agonist pioglitazone in mild Alzheimer disease. Neurobiol Aging. 2009.
112. Heneka MT, Landreth GE, Hull M. Drug insight: effects mediated by peroxisome proliferator-activated receptor-gamma in CNS disorders. Nat Clin Pract Neurol. 2007;3:496-504.

113. Hwang J, Kleinhenz DJ, Rupnow HL, et al. The PPARgamma ligand, rosiglitazone, reduces vascular oxidative stress and NADPH oxidase expression in diabetic mice. Vascul Pharmacol. 2007;46:456-462.
114. Pathan AR, Viswanad B, Sonkusare SK, Ramarao P. Chronic administration of pioglitazone attenuates intracerebroventricular streptozotocin induced-memory impairment in rats. Life Sci. 2006;79:2209-2216.
115. Park SA. A common pathogenic mechanism linking type-2 diabetes and Alzheimer's disease: evidence from animal models. J Clin Neurol. 2011;7:10-18.
116. Hay N. Interplay between FOXO, TOR, and Akt. Biochim Biophys Acta. 2011;1813:1965-1970.
117. Sebollela A, Freitas-Correa L, Oliveira FF, et al. Amyloid-beta oligomers induce differential gene expression in adult human brain slices. J Biol Chem. 2012;287:7436-7445.
118. Balaraman Y, Limaye AR, Levey AI, Srinivasan S. Glycogen synthase kinase 3beta and Alzheimer's disease: pathophysiological and therapeutic significance. Cell Mol Life Sci. 2006;63:1226-1235.
119. Hooper C, Markevich V, Plattner F, et al. Glycogen synthase kinase-3 inhibition is integral to long-term potentiation. Eur J Neurosci. 2007;25:81-86.
120. Hernandez F, Borrell J, Guaza C, Avila J, Lucas JJ. Spatial learning deficit in transgenic mice that conditionally over-express GSK-3beta in the brain but do not form tau filaments. J Neurochem. 2002;83:1529-1533.
121. Peineau S, Taghibiglou C, Bradley C, et al. LTP inhibits LTD in the hippocampus via regulation of GSK3beta. Neuron. 2007;53:703-717.
122. Zhao WQ, De Felice FG, Fernandez S, et al. Amyloid beta oligomers induce impairment of neuronal insulin receptors. FASEB J. 2008;22:246-260.
123. Hernandez F, Avila J. The role of glycogen synthase kinase 3 in the early stages of Alzheimers' disease. FEBS Lett. 2008;582:3848-3854.
124. Cao X, Wei Z, Gabriel GG, Li X, Mousseau DD. Calcium-sensitive regulation of monoamine oxidase-A contributes to the production of peroxyradicals in hippocampal cultures: implications for Alzheimer disease-related pathology. BMC Neurosci. 2007;8:73.
125. McLellan ME, Kajdasz ST, Hyman BT, Bacskai BJ. In vivo imaging of reactive oxygen species specifically associated with thioflavine S-positive amyloid plaques by multiphoton microscopy. J Neurosci. 2003;23:2212-2217.
126. Gotz ME, Fischer P, Gsell W, et al. Platelet monoamine oxidase B activity in dementia. A 4-year follow-up. Dement Geriatr Cogn Disord. 1998;9:74-77.
127. Grünblatt E, Schlosser R, Fischer P, et al. Oxidative stress related markers in the "VITA" and the centenarian projects. Neurobiol Aging. 2005;26:429-438.
128. Grünblatt E, Bartl J, Riederer P. The link between iron, metabolic syndrome, and Alzheimer's disease. J Neural Transm. 2010;118:371-379.
129. Kuroki T, Isshiki K, King GL. Oxidative stress: the lead or supporting actor in the pathogenesis of diabetic complications. J Am Soc Nephrol. 2003;14:S216-220.
130. Gao L, Mann GE. Vascular NAD(P)H oxidase activation in diabetes: a double-edged sword in redox signalling. Cardiovasc Res. 2009;82:9-20.
131. Nunes SF, Figueiredo IV, Pereira JS, et al. Monoamine oxidase and semicarbazide-sensitive amine oxidase kinetic analysis in mesenteric arteries of patients with type 2 diabetes. Physiol Res. 2010;60:309-315.

Publication list: Original Peer-reviewed publications

1. **Bartl J**, Meyer A., Brendler S, Riederer P, Grünblatt E (2012) Different effects of soluble and aggregated Amyloid β_{42} on gene/ protein expression and enzyme activity involved in insulin and APP pathways *under remission by Journal of Neural Transmission*

2. **Bartl J**, Monoranu CM, Wagner AK, Kolter J, Riederer P, Grünblatt E (2012) Alzheimer's disease and Type 2 Diabetes: two diseases, one common link? *World J Biol Psychiatry. in press*.

3. Grünblatt E, Geißler J., Jacob C P, Renner T, Müller M, **Bartl J**, Gross-Lesch S, Riederer P, Lesch KP, Walitza S, Gerlach M, Schmitt A(2012) Pilot study: potential transcription markers for adult attention-deficit hyperactivity disorder in whole blood. *J.ADHD. in press.*

4. **Bartl J,** Scholz CJ, Hinterberger M, Jungwirth S, Wichart I, Rainer MK, Kneitz S, Danielczyk W, Tragl KH, Fischer P, Riederer P, Grunblatt E (2011) Disorder-specific effects of polymorphisms at opposing ends of the Insulin Degrading Enzyme gene. *BMC Med Genet.; 12(1):151.*

5. **Bartl J**, Link P, Schlosser C, Gerlach M, Schmitt A, Walitza S, Riederer P, Grünblatt E (2010) Effects of methylphenidate: the cellular point of view. *ADHD Atten Def Hyp Disord* 2*(4):225-32*

6. Monoranu CM, Grünblatt E, **Bartl J**, Meyer A, Apfelbacher M, Keller D, Michel TM, Al-Saraj S, Schmitt A, Falkai P, Roggendorf W, Deckert J, Ferrer I, Riederer P (2010) Methyl- and acetyltransferases are stable epigenetic markers postmortem. *Cell Tissue Bank. 2011 Nov; 12(4):289-97.*

7. Grünblatt E, **Bartl J**, Zehetmayer S, Ringel TM, Bauer P, Riederer P, Jacob CP (2009) Gene expression as peripheral biomarkers for sporadic Alzheimer's disease..*J Alzheimers Dis*., 16(3):627-34.

8. Grünblatt E, Zehetmayer S, **Bartl J**, Löffler C, Wichart I, Rainer MK, Jungwirth S, Bauer P, Danielczyk W, Tragl KH, Riederer P, Fischer P (2009) Genetic risk factors and markers for Alzheimer's disease and/or depression in the VITA study. *J Psych Res*, Jan; 43(3):298-308.

9. Grünblatt E, Zander N, **Bartl J**, Jie L, Monoranu C.M, Arzberger T, Rivka R, Roggendorf W, Gerlach M, Riederer P (2007) Comparison analysis of gene expression patterns between sporadic Alzheimer's and Parkinson's disease. *J. Alzheimer Dis.* 12(4): 291-311.

10. Jacob CP, Koutsilieri E, **Bartl J**, Neuen-Jacob E, Arzberger T, Zander N, Ravid R, Roggendorf W, Riederer P, Grünblatt E (2007) Alterations in expression of glutamatergic transporters and receptors in sporadic Alzheimer's Disease *J. Alzheimer Dis.* 11(1):97-116

Reviews

1. Grünblatt, E., **Bartl J.,** Marinova Z, Walitza S (2012) In-vitro study methodologies to investigate genetic aspects and effects of drugs used in Attention-deficit Hyperactivity Disorder *submitted to JNT*

2. Riederer P, **Bartl J**, Laux G, Grünblatt E. (2011) Diabetes type II: a risk factor for depression-Parkinson-Alzheimer*? Neurotox Res. 19(2):253-65.*

3. Grünblatt E, **Bartl J**, Riederer P. (2010) The link between iron, metabolic syndrome, and Alzheimer's disease. *J Neural Transm.2011 Mar; 118(3):371-9.*

Abstracts:

1. Grünblatt E**, Bartl J**, Hofmann S, Borst A, Riederer P, Walitza S (2011) D/L threo-Methylphenidate enantiomers influence on catecholaminergic enzyme activities 27th Symposium of AGNP, Munich, October 5-8, 2011

2. **Bartl J.** , Wagner A.-K., Kolter J. , Riederer P. , Monoranu Camelia, Grünblatt E (2011) A post mortem histochemical study of Alzheimer patient with and without Diabetes type II 10th International Conference on AD/PD, Barcelona, March 9 - 13, 2011

3. **Bartl J**, Borst A, Iuhos D, Riederer P, Salkovic-Petrisic M , Walitza S, Grünblatt E (2011) Insulin resistance, hypertension and Attention Deficit Hyperactive Disorder: is there a link? Swiss Society of Neuroscience Annual Meeting. Basel. 26th March, 2010

4. **Bartl J**, Grünblatt E, Gerlach M, Riederer P (2009) Effect of Methylphenidate - a point of view from a cell 10th Congresses of WFSBP , Paris

5. **Bartl J,** Grünblatt E, Gerlach M, Riederer P, Mori T, Ozawa H (2007) Methylphenidate effects on cell growth and maturation in neuronal stem cells. 39th Danube Symposium and 1st International Congress on ADHD, June 2-5 2007, Würzburg, Germany.

6. Grünblatt E, Mandel S, Jacob C, **Bartl J**, Zander N, Ravid R, Arzberger T Müller T, Jost WH, Roggendorf W, Youdim MBH, Riederer P (2007) Gene chip analysis in post mortem brains and whole blood of PD and AD: A comparison. IBRO World Congress of Neuroscience, July 12-17, 2007, Melbourne, Australia.

7. Grünblatt E, **Bartl J**, Zander N, Ravid R, Arzberger T, Roggendorf W, Jacob C, Riederer P (2006) Gene-expression profiling in sporadic Alzheimer's disease Patients: Post mortem vs. blood samples. The Second Dual Congress on Psychiatry and Neurosciences., December 7-10, 2006, Athens, Greece

Zurich, 30th of May, 2012