

PhD Thesis

Mechanisms governing changes in cerebrovascular insulin signaling in Alzheimer's disease

submitted by

Chaitanya Chakravarthi Gali

for the Academic Degree of

Doctor of Philosophy (PhD)

at the

Medical University of Graz, AUSTRIA

**Otto Loewi Research Center for Vascular Biology, Immunology
and Inflammation, Immunology and Pathophysiology**

under the supervision of

Assoz. Prof. PD Mag. Dr. Ute Panzenboeck

2019

DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis and agreed to publish the data in this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

The major part of investigation presented in this thesis has been summarized and published in:

Gali CC, Fanaee-Danesh E, Zandl-Lang M, Albrecher NM, Tam-Amersdorfer C, Stracke A, Sachdev V, Reichmann F, Sun Y, Avdili A, Reiter M, Kratky D, Holzer P, Lass A, Kandimalla KK, Panzenboeck U. Amyloid-beta impairs insulin signaling by accelerating autophagy-lysosomal degradation of LRP-1 and IR-beta in blood-brain barrier endothelial cells in vitro and in 3XTg-AD mice. *Mol Cell Neurosci*. 2019 Jul 2;99:103390. (1) DOI: 10.1016/j.mcn.2019.103390

List of all co-authors to this publication:

- *Division of Immunology and Pathophysiology, Otto Loewi Research Center, Medical University of Graz, Graz, Austria:*
Chaitanya Chakravarthi Gali, Elham Fanaee-Danesh, Martina Zandl-Lang, Nicole Maria Albrecher, Carmen Tam-Amersdorfer, Anika Stracke, Yidan Sun, Afrim Avdili, Marielies Reiter and Ute Panzenboeck (corresponding author)
- *Molecular Biology and Biochemistry, Gottfried Schatz Research Center, Medical University of Graz, Graz, Austria:*
Vinay Sachdev and Dagmar Kratky
- *Pharmacology, Otto Loewi Research Center for Vascular Biology, Immunology and Inflammation, Medical University of Graz, Graz, Austria:*
Florian Reichmann and Peter Holzer
- *Institute of Molecular Biosciences, University of Graz, Graz, Austria:*
Achim Lass

- *College of Pharmacy, Department of Pharmaceutics, University of Minnesota, Minneapolis, MN, USA:*
Karunya K. Kandimalla

Graz, August 2019

Chaitanya Chakravarthi Gali, MSc.

ACKNOWLEDGEMENTS

Foremost, I would like to convey my deepest gratitude to my supervisor Professor **Ute Panzenboeck** for considering me and having faith in my ability to succeed as a PhD student. I sincerely acknowledge her consistent support, constructive criticism and thought provocation to enable motivation and enthusiasm towards research and in writing my manuscript and thesis. Apart from my research project, she encouraged me to participate in many collaborative and industrial projects, which equipped me with industrial acumen, being skillful at understanding the problem on the go and the ability to execute the projects with better success.

I extend my sincere appreciation towards my thesis committee members Professor **Klaus Groschner** (Gottfried Schatz Research Center, Medical University of Graz) and Professor **Achim Lass** (Institute of Molecular Biosciences, University of Graz) for their invaluable suggestions and comments, which helped me to construct my research the way it is today.

Besides my advisor, I would like to extend my sincere gratitude to Professor **Achim Lass** for his consistent encouragement and mentorship throughout my journey as a PhD student. I sincerely regard his presence when needed and during the toughest parts of my journey as a PhD student. I would like to express my deepest gratitude to Professor **Karunya K Kandimalla** for inviting me to pursue my research stay in his lab at University of Minnesota and his mentorship during my stay at University of Minnesota (Minneapolis) and Mayo Clinic (Rochester).

I sincerely thank my fellow research students (**Yidan Sun, Elham Fanaee-Danesh, Martina Zandl-Lang, Victoria Zyulina, Christina Passegger, Tommaso Sconocchia, Marielies Reiter, Meekha George** and **Alexandra Kober**) at the institute for their constant support at work and maintaining a supportive and friendly environment within and outside the institute.

My sincere thanks to fellow technicians (**Anika Stracke, Nicole Maria Albrecher** and **Carmen Tam-Amersdorfer**) and staff members (**Karin Schramke, Waltraud Huber** and **Magdolna Mayer**) for their tremendous effort and support to keep up with our research and maintaining the harmony at the institute.

I take this opportunity to acknowledge the support and mentorship of my fellow researchers at University of Minnesota (Department of Pharmaceutics; **Suresh Kumar Swaminathan**) and Mayo Clinic (Radiology; **Vidur Sharma, Geoffry Curran, Tyler Bruinsma** and **Paul H. Min**) for teaching me high-end imaging and surgical techniques, during my research stay in the USA. I extend my sincere gratitude to *Austrian Marshall Plan Foundation* for financially supporting me to undertake research stay in the USA.

I would like to express my deepest gratitude and to be forever grateful to DK-MCD (*Metabolic and Cardiovascular Disease*) PhD program, Medical University of Graz, FWF (*Fonds zur Förderung der wissenschaftlichen Forschung*) and the organizers of DK-MCD for funding (Grants; W1226 and P24783 to **Ute Panzenboeck**) my research and giving me the opportunity to pursue PhD in Graz. I would like to acknowledge FWF (Grant; I3535 to **Achim Lass**), PhD program “Molecular Medicine” of Medical University of Graz and BioTechMed-Graz (Flagship Project “Lipases and Lipid Signaling” to **Achim Lass** and **Dagmar Kratky**) for sponsoring my work.

I would like to express my sincere gratitude to **Karin Osibow** (*Project coordinator; DK-MCD*) for her invaluable support to PhD students with courses, administrative works, traveling, housing and legal works. Without her guidance, we as PhD students are lost, and I sincerely appreciate her efforts to guide us through the PhD program.

Lastly but More importantly, I would like to extend my heartfelt gratitude and love to my family members (**parents and brother**) and close friends (**Shirony Nicholson Puthenveedu, Meghana Somlapura, Niroj Shrestha** and **Chintan NC Koyani**) for their love and consistent support throughout my journey, without which I am not the person today.

ABBREVIATIONS

3XTg-AD, APP_{swe}, PS1_{M146V}, tau_{P301L};
4E-BP1, 4E-binding protein 1;
ABCB1/Pgp, P-glycoprotein 1
AD, Alzheimer's disease;
ADAM10, α -secretase/ a disintegrin and metalloproteinase;
APN/CD13, aminopeptidase N;
apoE, apolipoprotein E;
apoJ, apolipoprotein J;
APP, amyloid precursor protein;
APP_{swe}, swedish mutation of amyloid precursor protein;
ATG, autophagy-related protein;
A β , amyloid beta peptide;
BACE1, beta-secretase 1;
BBB, blood-brain barrier;
BCA, bicinchoninic acid assay;
BCEC, brain capillary endothelial cells;
BM, barnes maze;
BSA, bovine serum albumin;
CAA, cerebral amyloid angiopathy;
CNS, central nervous system;
CSF, cerebrospinal fluid;
CypA, cyclophilin A;
DEA, diethylamine;
DEANB, diethylamine neutralizing buffer;
EDTA, ethylenediaminetetraacetic acid;
EGTA, ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid;
ER, endoplasmic reticulum;
FA, formic acid;
FANB, formic acid neutralization buffer;
FDG-PET, fluorodeoxyglucose F18 - positron emission tomography;
Foxo, forkhead box O;
GLP-1, glucagon-like peptide 1;

GLUT1, glucose transporter;
HCl, hydrochloric acid;
HFD, high-fat high-cholesterol diet;
HFIP, hexafluoro-2-propanol;
HOPS, homotypic fusion and protein sorting;
HPRT-1, hypoxanthine phosphoribosyltransferase 1;
HSP27, heat shock protein 27;
IB, immunoblotting;
IBA1, ionized calcium-binding adapter molecule 1;
IDE, insulin-degrading enzyme;
IF, immunofluorescent;
IGF-1, insulin /insulin-like growth factor 1
IR(s), insulin receptor(s);
IRS-1, insulin receptor substrate-1;
IR- β , insulin receptor beta;
JNK, c-Jun N-terminal kinase;
KO, knock out;
LAMP-1, lysosomal-associated membrane protein 1;
LC3B, microtubule-associated proteins 1A/1B light chain 3B;
LOAD, late onset Alzheimer's disease;
LRP-1, low-density lipoprotein receptor-related protein-1;
MAP2, microtubule-associated protein 2;
MAPK, mitogen-activated protein kinase;
mBCEC, murine brain capillary endothelial cells;
MCI, mild cognitive impairment;
MFSD2a, major facilitator superfamily domain-containing protein 2;
mm, mus musculus;
MMP's, matrix metalloproteinases;
MMP-9, matrix metalloproteinase-9;
mTORC2, mammalian target of rapamycin complex 2;
Na₂HPO₄, sodium phosphate dibasic;
NaN₃, sodium azide;
NC, nitrocellulose;
NEFA, non-esterified fatty acids;

NFT, neurofibrillary tangles;
NTC, non-targeting control;
NTg, non-transgenic;
OGTT, oral glucose tolerance test;
PAS, phagophore assembly site;
pBCEC, porcine brain capillary endothelial cells;
PDGFR- β , platelet-derived growth factor receptor beta;
PDK-1, 3-phosphoinositide-dependent protein kinase 1;
PECAM/CD31, platelet endothelial cell adhesion molecule;
PET, positron emission tomography;
PFA, paraformaldehyde;
PI3-K, phosphoinositide 3-kinase;
PICALM, phosphatidylinositol-binding clathrin assembly protein;
PIP2, phosphorylating phosphatidylinositol 4,5-bisphosphate;
PIP3, phosphatidylinositol (3,4,5)-triphosphate;
PKB, AKT/protein kinase B;
PKC, protein kinase C;
PPAR γ , proliferator-activated receptor- γ ;
PSMD4, 26S proteasome non-ATPase regulatory subunit 4;
PVDF, polyvinylidene difluoride;
RAGE, receptor for advanced glycated end products;
RT, room temperature;
RT-qPCR, quantitative Real time PCR;
S6K, p70 ribosomal S6 kinase;
SF, serum free;
SGK, serum- and glucocorticoid-induced protein kinase;
sLRP-1, soluble low-density lipoprotein receptor-related protein-1;
SorLA, sortilin-related receptor;
SREBP-1, sterol regulatory element-binding protein-1;
ss, sus scrofa;
SYP, synaptophysin;
T2DM, type-2 diabetes mellitus;
TGN, trans-golgi network;
THB, tissue homogenization buffer;

TMB, tetramethylbenzidine;
TNF- α , tumor necrosis factor-alpha;
TRAPP, transport protein particle;
TSC-2, tuberous sclerosis complex protein-2;
UPS, ubiquitin-proteasome system;
ZO1, zonula occludens protein-1;
 α 2MR, α 2-macroglobulin receptor;

TABLE OF CONTENTS

DECLARATION	1
ACKNOWLEDGEMENTS.....	3
ABBREVIATIONS	5
LIST OF FIGURES.....	14
LIST OF TABLES	16
ABSTRACT	17
KURZFASSUNG	18
1. INTRODUCTION	20
1.1 Alzheimer's disease and its prevalence	20
1.2 Amyloid- β production	22
1.3 Amyloid- β catabolism in brain and periphery.....	25
1.4 Insulin production, function and pathways regulating insulin signaling....	28
1.5 Type-2 diabetes mellitus, a risk factor for Alzheimer's disease?	35
1.6 Brain insulin signaling in Alzheimer's disease	37
1.7 Association between brain insulin receptor- β and low-density lipoprotein related protein-1	40
1.8 The blood-brain barrier and its function.....	42
1.9 Amyloid- β clearance pathways at the BBB.....	45
1.10 Pathways regulating intracellular A β degradation and protein homeostasis.....	47
2. HYPOTHESIS AND AIMS	49
3. MATERIALS AND METHODS	50
3.1 Chemicals and reagents used for cell culture.....	50
3.1.1 Phosphate buffer saline (PBS).....	50
3.1.2 Collagenase/dispase solution	51
3.1.3 Dextran solution	51

3.1.4	Percoll® biphasic solution	51
3.1.5	Hydrocortisone solution (50 µg/ml)	51
3.1.6	Collagen G solution for coating flasks and cell culture plates	51
3.1.7	Preparation medium.....	51
3.1.8	Plating medium A.....	51
3.1.9	Plating medium B.....	52
3.1.10	Serum free (SF) medium	52
3.1.11	DMEM/Ham's F12 medium.....	52
3.1.12	Insulin stock solution (10 mM)	52
3.1.13	Amyloid-β peptide stock solution	52
3.1.14	MG132 stock solution (10 mM).....	52
3.1.15	Chloroquine stock solution (30 mM)	52
3.2	Chemicals and solutions used for RNA isolation, cDNA synthesis and quantitative Real time PCR (RT-qPCR)	53
3.2.1	RNA isolation from mouse tissue or endothelial cells	53
3.2.2	cDNA preparation	55
3.2.3	RT-qPCR	55
3.3	Silencing of LRP-1 and IR-β in pBCEC	57
3.4	Protein isolation from cell and tissue lysates.....	59
3.5	Determining protein concentration of cell or tissue lysates.....	59
3.6	Western blotting (WB)	59
3.6.1	Transfer buffer 10x (Towbin buffer).....	60
3.6.2	Protein lysis buffer (PLB)	61
3.6.3	Blocking buffer	61
3.6.4	Sample preparation and SDS-polyacrylamide gel electrophoresis (PAGE)	61
3.6.5	Protein transfer from gel to membrane	62
3.6.6	Ponceau S staining of proteins	62

3.6.7	Membrane blocking.....	62
3.6.8	Primary antibody incubation.....	62
3.6.9	Washing the blots	63
3.6.10	Secondary antibody incubation.....	63
3.6.11	Signal detection on Western blots and semi-quantitative analysis ...	63
3.7	Animals & housing.....	64
3.8	Genotyping.....	65
3.9	Feeding timeline.....	66
3.10	Oral glucose tolerance test	68
3.11	Barnes Maze study	69
3.12	Harvesting primary organs and blood	70
3.13	Plasma isolation.....	70
3.14	Quantitative analysis of cholesterol, triglycerides and non-esterified fatty acids in plasma samples	70
3.15	Quantification of plasma insulin levels	71
3.16	Extraction of soluble and insoluble A β from mouse brain	72
3.16.1	Reagents required	73
3.16.2	Diethylamine neutralizing buffer (DEANB) or 0.5 M Tris-HCl (pH 6.8)..	74
3.16.3	Procedure for DEA and FA extraction of A β products.....	74
3.17	A β ₁₋₄₂ and A β ₁₋₄₀ ELISA	74
3.17.1	Buffers required for ELISA assay.....	75
3.17.1.1	Standard reconstitution buffer.....	75
3.17.1.2	Washing buffer.....	75
3.17.1.3	1X Anti-Rabbit IgG HRP solution	75
3.17.2	ELISA assay and measurement	75
3.18	Isolation of murine brain microvasculature.....	76
3.19	Isolation of porcine brain capillary endothelial cells (pBCEC)	77

3.20	Congo red staining to detect amyloid plaques and fibril deposits in mouse brain sections.....	78
3.20.1	Reagents required for Congo red staining	78
3.20.1.1	Modified Weigert's iron hematoxylin	78
3.20.1.2	0.5% acid alcohol.....	78
3.20.1.3	2.5% sodium chloride (NaCl-) alcohol.....	78
3.20.1.4	1% sodium hydroxide solution	79
3.20.1.5	Alkaline Congo red	79
3.20.1.6	Congo red staining procedure and microscopy	79
3.21	Immunofluorescent staining	79
3.21.1	IF staining of snap frozen mouse brain sections.....	79
3.21.2	Double-label IF staining of heat fixed brain microvessels	80
3.21.3	IF staining of pBCEC	80
3.21.4	Detection of IF stainings	81
3.22	Cerebromicrovascular LC3B dot coverage quantification	82
3.23	Statistical analyses	82
4.	RESULTS	84
4.1	Part 1: Impact of 3XTg-AD transgenes and HFD feeding on cerebral A β accumulation, memory, and peripheral and central insulin signaling	84
4.1.1	Genotyping of 3XTg-AD mice carrying mutations for human APP _{swe} , PS1 _{M146V} and tauP301L	84
4.1.2	HFD feeding enhanced body weight gain and worsened glucose tolerance in NTg and 3XTg-AD mice.....	85
4.1.3	AD transgenes and HFD feeding modulate plasma insulin and lipid profile in 3XTg-AD and NTg mice.	88
4.1.4	HFD feeding aggravates A β burden in 3XTg-AD mouse brain	90
4.1.5	Effect of HFD feeding on memory impairment in male and female 3XTg-AD mice	92

4.1.6	Effects of HFD feeding and AD transgene on liver insulin signaling in 3XTg-AD and HFD fed mice.	95
4.1.7	Altered brain insulin signaling in 3XTg-AD and HFD fed mice	96
4.1.8	LRP-1 and IR- β signature and their interaction in the mouse cerebrovascular endothelium	100
4.1.9	Impaired cerebrovascular endothelial LRP-1 and IR- β levels in 3XTg-AD mice.....	102
4.1.10	Absence of cerebrovascular pro-fibrillary/fibrillary A β deposits or CAA in 9-month-old male and female HFD fed 3XTg-AD mice.....	104
4.1.11	Elevated cerebral and cerebrovascular autophagy in 3XTg-AD mice... ..	106
4.2	Part 2: Implications of A β burden on blood-brain barrier LRP-1 and IR- β regulation, and insulin signaling <i>in vitro</i>	110
4.2.1	Insulin signaling pathways in pBCEC.....	110
4.2.2	A β treatment <i>in vitro</i> impairs IR- β and LRP-1 protein levels and their interaction in primary pBCEC	112
4.2.3	A β treatment <i>in vitro</i> impairs insulin signaling in pBCEC	113
4.2.4	A β treatment accelerates LRP-1 and IR- β degradation via autophagy-lysosomal induction in pBCEC	115
4.2.5	LRP-1 silencing disrupts IR- β levels and insulin signaling in pBCEC.	117
5.	DISCUSSION	120
6.	BIBLIOGRAPHY	127

LIST OF FIGURES

Figure 1: Prevalence of AD in the United States according to a 2016 report	21
Figure 2: Clinical and pathological development of Alzheimer's disease.	23
Figure 3: Mechanism of Amyloid- β genesis in a neuronal cell.....	25
Figure 4: Amyloid- β clearance pathways.....	27
Figure 5: Biosynthesis of insulin from proinsulin and preproinsulin	29
Figure 6: Possible pathways governing transcytosis of insulin across the endothelial barrier	31
Figure 7: Pathways governing insulin signaling	34
Figure 8: Correlation between T2DM, brain insulin resistance, and AD	37
Figure 9: Distinctions in brain insulin signaling in healthy and AD subjects.....	39
Figure 10: Prototype of LRP-1 regulating insulin signaling and glucose metabolism in neuronal cells	42
Figure 11: Structure of the BBB.....	43
Figure 12: Diagram showing key transport properties of the BBB	44
Figure 13: Pictorial representation of A β clearance pathways at the BBB.....	46
Figure 14: Protein degradation pathways (autophagy-lysosomal processing and ubiquitin proteasome system) under healthy and AD conditions	48
Figure 15: Diagrammatic representation of Barnes Maze with quadrants.	69
Figure 16: Confirmation of mutations for human APP _{Swe} and PS1 _{M146V} genes in 3XTg-AD mice	85
Figure 17: Impact of HFD feeding on BW and glucose tolerance in male and female 3XTg-AD and NTg mice.	87
Figure 18: Impact of HFD feeding on plasma lipids and insulin levels in 3XTg-AD and NTg mice.	89
Figure 19: Effects of AD transgene, gender and HFD feeding on cerebral A β burden.	91
Figure 20: Effects of AD transgene, gender and HFD on anxiety and memory in mice.....	94
Figure 21: Effects of AD transgene, gender and HFD on liver insulin signaling and IR- β /LRP-1 levels.	96
Figure 22: Modified brain insulin signaling and LRP-1 levels in 3XTg-AD and HFD fed mice.....	99

Figure 23: Purity of isolated murine brain microvasculature and double IF staining of LRP-1 and IR- β in mouse cerebromicrovascular endothelium.	102
Figure 24: Effect of AD transgenes, gender and HFD on cerebromicrovascular endothelial LRP-1 and IR- β levels in 3XTg-AD and NTg mice.	103
Figure 25: Effect of AD transgenes and HFD on cerebrovascular A β deposition in 3XTg-AD mice.	105
Figure 26: Elevated cerebrovascular and brain autophagy in 3XTg-AD mice. ...	108
Figure 27: Insulin signaling apparatus is conserved in pBCEC.	111
Figure 28: Effects of A β isoforms on LRP-1 and IR- β protein levels and their interaction in pBCEC.	112
Figure 29: Effects of A β_{1-40} or A β_{1-42} peptides on insulin signaling in pBCEC.	114
Figure 30: Effects of A β_{1-40} treatment on LRP-1/ IR- β levels and LAMP-1 in pBCEC.	116
Figure 31: Immunofluorescent staining depicting the effect of lysosomal and proteasomal inhibitors on A β_{1-40} induced LRP-1 and IR- β impairment and LAMP-1 induction in pBCEC.	117
Figure 32: Role of LRP-1 in BBB endothelial IR- β regulation and insulin signaling.	118
Figure 33: Diagrammatic representation of processes involved in A β mediated degradation of receptors important for insulin signaling at the BBB.	126

LIST OF TABLES

Table 1: Cell culture reagents and chemicals.....	50
Table 2: Materials used for RNA isolation, cDNA synthesis and RT-qPCR.....	53
Table 3: High-Capacity cDNA Reverse Transcription (master mix composition) ..	55
Table 4: Reverse transcription thermal cycling conditions.....	55
Table 5: Primers used for RT-qPCR.....	56
Table 6: Real-time PCR mix	57
Table 7: RT-qPCR program conditions	57
Table 8: siRNA sequences for LRP-1 and IR- β mRNA	58
Table 9: Buffers and reagents used for WB.....	59
Table 10: composition of Towbin buffer.....	60
Table 11: Wash buffer (TBS-T) (pH 8.5)	61
Table 12: List of antibodies used for western blotting.....	63
Table 13: Genotyping for APP.....	65
Table 14: Genotyping for PS1	65
Table 15: Bste II digest preparation.....	66
Table 16: Cycle conditions used for DNA amplification	66
Table 17: Composition of high fat diet (Ssniff® diets).....	66
Table 18: Assay conditions for triglycerides and/or cholesterol	70
Table 19: Assay conditions for NEFA.....	71
Table 20: Insulin ELISA method	72
Table 21: Materials and equipment required for A β extraction from mouse brain	72
Table 22: Materials and equipment required for preparing DEA solution	73
Table 23: Formic acid neutralization buffer (FANB) composition.....	73
Table 24: Tissue homogenization buffer (THB) composition	73
Table 25: DEANB composition	74
Table 26: Contents of A β ₁₋₄₂ and A β ₁₋₄₀ ELISA kits	75
Table 27: List of antibodies used for IF staining	81

ABSTRACT

Cerebral insulin signaling impairment has been identified in Alzheimer's disease (AD) subjects and AD mouse models. In brain, insulin signaling is key for neuronal growth, memory consolidation and long-term potentiation. Low-density lipoprotein receptor-related protein-1 (LRP-1) was shown to modulate insulin signaling by dimerizing with insulin receptor beta (IR- β) upon insulin stimulation, and LRP-1 deletion is known to impair IR- β receptor levels thereby hindering insulin mediated responses in mice. Both LRP-1 and IR- β are expressed in cerebrovascular endothelial cells which constitute the blood-brain barrier (BBB). Recent evidence shows that insulin regulates surface expression of LRP-1 in hepatocytes and at the BBB and could possibly modulate A β clearance from the brain and periphery. Even though several studies reported changes in brain insulin signaling in AD, cerebrovascular endothelial modulation of insulin signaling, mediated by IR- β and LRP-1 in AD is not well understood. In the present thesis we found a significant impairment in brain and cerebrovascular IR- β and LRP-1 levels in 9-month-old male and female 3XTg-AD (PS1^{M146V}, APP^{Swe}, and tau^{P301L}) mice when compared to age matched NTg mice, which in further consequence affected insulin mediated signaling response pathways. Alterations observed in brain and cerebrovascular IR- β and LRP-1 levels correlated with elevated LC3B levels in 3XTg-AD mice indicating activation of autophagy. High-fat diet (HFD) feeding reduced cerebral and hepatic LRP-1 levels and enhanced cerebral A β burden without showing considerable effects on cerebrovascular LRP-1 and IR- β levels. Subsequent *in vitro* studies using porcine brain capillary endothelial cells (pBCEC) revealed that treatment with A β ₁₋₄₀ or A β ₁₋₄₂ impaired LRP-1 and IR- β receptor levels and hampered their interaction in BCEC, thereby plummeting insulin mediated signaling. Subsequent mechanistic exploration confirmed that A β treatment enhanced the autophagy-lysosomal degradation of LRP-1 and IR- β receptors in pBCEC. Using siRNA approach in pBCEC, we identified that LRP-1 deletion post-translationally hampered IR- β levels and in turn reduced insulin-mediated response pathways at the BBB. Our findings indicate that cerebral A β burden in AD may accelerate LRP-1 and IR- β degradation in BCEC thereby weakening cerebrovascular (and cerebral) insulin effects.

KURZFASSUNG

Eine Beeinträchtigung des zerebralen Insulinsignalweges wurde sowohl bei Patienten der Alzheimer Krankheit (AD) als auch in AD Mausmodellen beobachtet. Der Insulinsignalweg spielt im Gehirn eine entscheidende Rolle für das Wachstum neuronaler Zellen, die Festigung von Erinnerungen und die Langzeitpotenzierung. Das Low-density Lipoprotein Receptor related Protein-1 (LRP-1) moduliert in Folge eines Stimulus durch Insulin den Insulinsignalweg durch eine Dimerisierung des Insulinrezeptors- β (IR- β). Von Mausmodellen ist bekannt, dass eine Deletion von LRP-1 die Menge von IR- β vermindert und dadurch der Insulinsignalweg behindert wird. Die zerebromikrovaskulären Endothelzellen, welche die Blut-Hirn-Schranke (BBB) darstellen, exprimieren sowohl LRP-1 als auch IR- β . Jüngste Studien haben gezeigt, dass Insulin in Leberparenchymzellen und auch an der BBB, den Expressionsspiegel von LRP-1 reguliert und daher möglicherweise den Abtransport von A β sowohl aus dem Gehirn wie auch in der Peripherie moduliert. Obwohl von einigen Studienautoren Modifikationen des Insulinsignalweges bei AD beobachtet wurden, sind die diesbezüglichen Veränderungen an den zerebrovaskulären Endothelzellen durch IR- β und LRP-1 im Verlauf von AD noch nicht zur Gänze aufgeklärt. In der vorliegenden Abschlussarbeit zeigen wir, dass es bei neun Monate alten männlichen und weiblichen 3XTg-AD Mäusen (PS1_{M146V}, APP_{Swe}, and tau_{P301L}) verglichen mit gleichaltrigen nichttransgenen Tieren, im Gesamtgehirn und der Zerebromikrovaskulatur zu einer signifikanten Reduktion der Expression von IR- β and LRP-1 kommt. Diese Beeinträchtigung wirkt sich in weiterer Folge auf die dem Insulinsignalweg nachgeschalteten Prozesse aus. Die veränderte Expressionshöhe von IR- β und LRP-1 in Gehirn und Zerebromikrovaskulatur der 3XTg-AD Mäuse korrelierte mit einer Erhöhung der LC3B Expression, was auf eine Aktivierung der Autophagie hindeutet. Fettreiche Ernährung (HFD) der Versuchstiere führte zu einer Reduktion der Expression von LRP-1 sowohl im Gehirn als auch in der Leber und erhöhte die A β Last, ohne nennenswerte Auswirkungen auf die Höhe der zerebromikrovaskulären LRP-1 und IR- β Expression zu haben. Weiterführende *in vitro* Versuche mit Schweinehirnkapillarendothelzellen (pBCEC) zeigten, dass eine Behandlung mit A β ₁₋₄₀ or A β ₁₋₄₂ zu veränderten LRP-1 und IR- β Level führt, deren Interaktion beeinträchtigt wird und es zum Absturz der weiterführenden Insulin-medierten Signalkaskade kommt. Durch weitere mechanistische Versuche konnten wir bestätigen,

dass es durch eine Behandlung der pBCEC mit A β zu einem verstärkten autophago-lysosomal Abbau von LRP-1 und IR- β kommt. Mit Hilfe von siRNA Versuchen konnten wir in pBCEC zeigen, dass es in Folge einer Deletion von LRP-1 zu einer post-translationalen Reduktion des IR- β Levels kommt und dies in weiterer Folge die Insulin mediierten Effekte an der BBB unterdrückt. Unsere Ergebnisse legen nahe, dass bei AD die A β Last im Hirn die LRP-1 und IR- β Degradation beschleunigt und es dadurch in der Mikrozerebrovaskulatur (und im Gesamthirn) zu einer Abschwächung der Wirkung von Insulin kommt.

1. INTRODUCTION

1.1 Alzheimer's disease and its prevalence

Alzheimer's disease (AD) is the most prominent form of dementia, begins with poorly recognized failure of memory and slowly becomes more severe and eventually incapacitating (2). The median duration of illness was found to be 8-10 years but the clinically relevant symptomatic phase is preceded by prodromal phase, which typically extends over two decades (3). Symptoms comprise memory disorientation, confusion, poor judgement, language disturbance, agitation, withdrawal, hallucinations and eventually will lead to death primarily due to symptoms like cachexia, pulmonary embolism, cardiovascular disorders, pneumonia and dehydration (4, 5). Over 95% of all reported AD cases are late onset (age >60-65 years) while less than 5% are considered as early onset (age <60-65 years) (4). In the USA alone, an estimated 5.4 million people of all age groups have AD in 2016, out of which, 5.2 million individuals are of age 65 years and older and roughly 200,000 individuals were reported to be under 65 years of age. In the United States, every one out of nine individuals with age above 65 years has AD and about one-third of individuals with age 85 years or above has AD (**Fig. 1A**) (6). Elderly women are more susceptible to AD than elderly men, even though the critical reasons behind the gender susceptibility to AD are not well understood. Some studies report that AD risk is higher in women is associated with biological or genetic factors or life experiences (6). Two-thirds of Americans with AD are women, out of 5.2 million people with age 65 years and above, diagnosed with AD, 3.3 million were women and 1.9 million were men (**Fig. 1B**). In the United States of America (USA), AD is officially listed as the 6th leading cause of death for the individuals with age 65 years and older (**Fig. 1C**) (6).

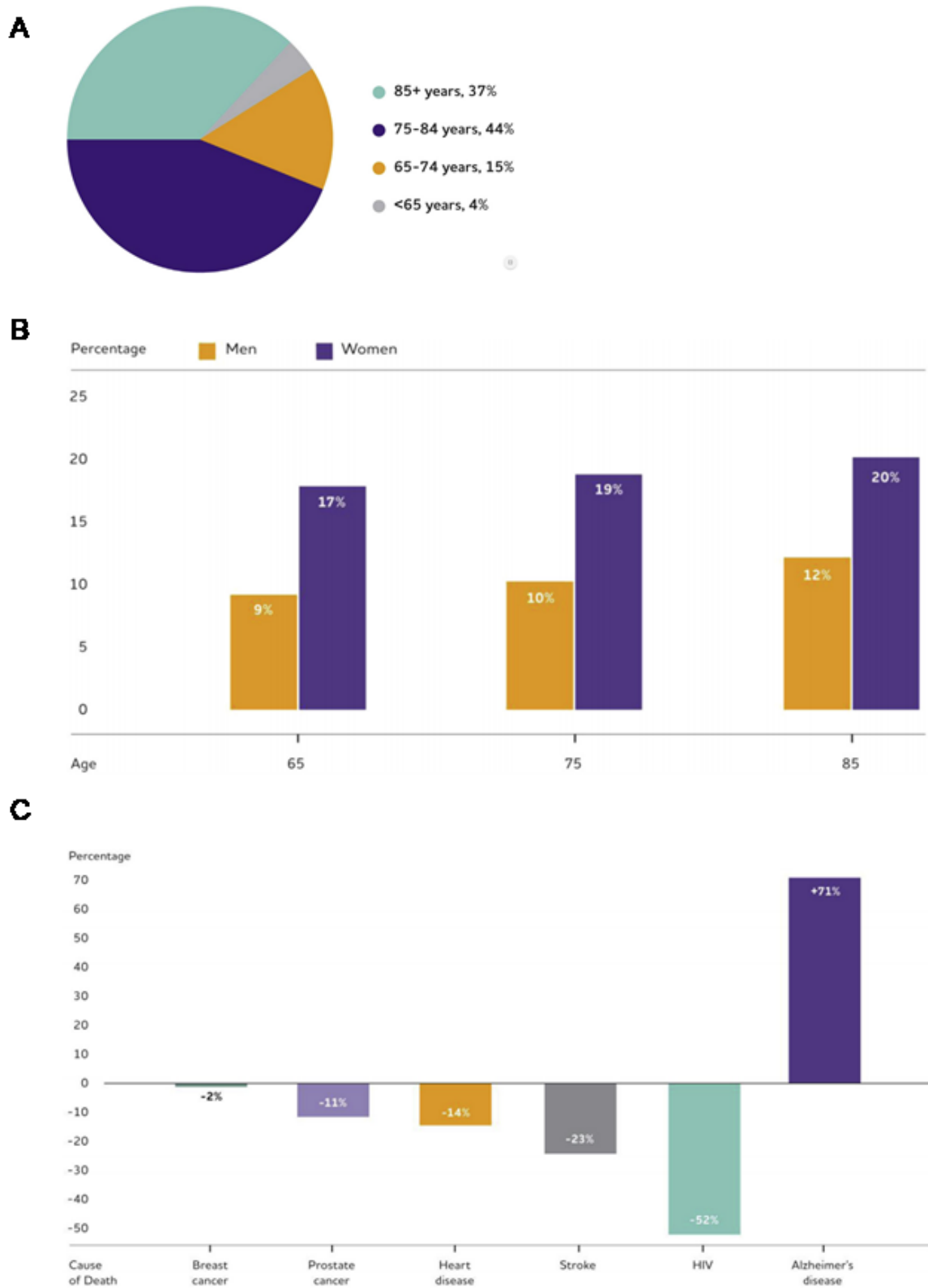


Figure 1: Prevalence of AD in the United States according to a 2016 report

A) Age of people with Alzheimer's disease in the United States, 2016. B) Estimated lifetime risk for Alzheimer's, by age and sex, from the Framingham Study. C) Percentage changes in selected

causes of death (all ages) between 2000 and 2013. [Reproduced from 2016 Alzheimer's disease facts and figures (Fig. 1,3&5) with the permission of Alzheimers Dement (6)]

1.2 Amyloid- β production

With advancements in research, in the past three decades many efforts were made in understanding the pathogenesis of AD. The scientific community strongly believes that AD is a disorder of abnormal protein aggregation and accumulation in various regions of the brain. Histopathological features of AD are extracellular A β plaques, intracellular neurofibrillary tangles (NFT) and amyloid angiopathy at post-mortem examination (**Fig. 2A&B**) (4).

The precursor of A β , the amyloid precursor protein (APP) belongs to the family of membrane proteins called amyloid precursor-like proteins (APLP)¹ and APLP², commonly expressed in all mammals (7). Alternate splicing of the APP transcript produces eight isoforms of which three are most common: the 695 amino acid (aa) form which is expressed predominantly in the CNS, and the 751 and 770 aa forms which are ubiquitously expressed (8). In neuronal cells, subsequent to endoplasmic reticulum (ER) and Golgi sorting, APP is transported to the axon, where it is conveyed to synaptic terminals via fast axonal transport. APP processing occurs primarily at the cell surface and in the trans-golgi network (TGN) (7). APP can be cleaved directly by α -secretase/A disintegrin and metalloproteinase (ADAM10) and then by γ -secretase, a sequential process that generates non-amyloidogenic fragments largely at the plasma membrane. Alternatively, APP fragments are reinternalized in clathrin-coated pits into another endosomal compartment, harboring the proteases beta-secretase 1/2 (BACE1/2) and γ -secretase, which sequentially generate peptides of various lengths mainly A β ₁₋₄₀ and A β ₁₋₄₂ with amyloidogenic properties (**Fig. 3**) (9-12). In humans, A β production from APP occurs in a variety of cell types including brain cells like neurons, astrocytes and microglia, but also in peripheral tissues, such as the adrenal glands, kidney, heart, liver, spleen, pancreas, muscles, and several blood and endothelial cells. However, A β production in brain outweighs the A β production in peripheral organs. Important distinctions have been identified between the central and peripheral pools of A β species. Importantly, A β ₁₋₄₂ was identified to be the most neurotoxic species prone to aggregation and is predominantly found in the brain (13, 14). In contrast, A β ₁₋₄₀ is the most abundant iso-

form present in the peripheral compartment. In part, these differences can be attributed to the expression of different APP isoforms in the brain and periphery. Other possibilities include differential processing of APP in the peripheral compartment when compared to the central compartment, and/or predominant α -secretase mediated APP processing as opposed to β -secretase APP processing in neuronal cells. Lastly, availability or abundance of A β -binding proteins in central and peripheral compartments may contribute to A β metabolism and clearance (14).

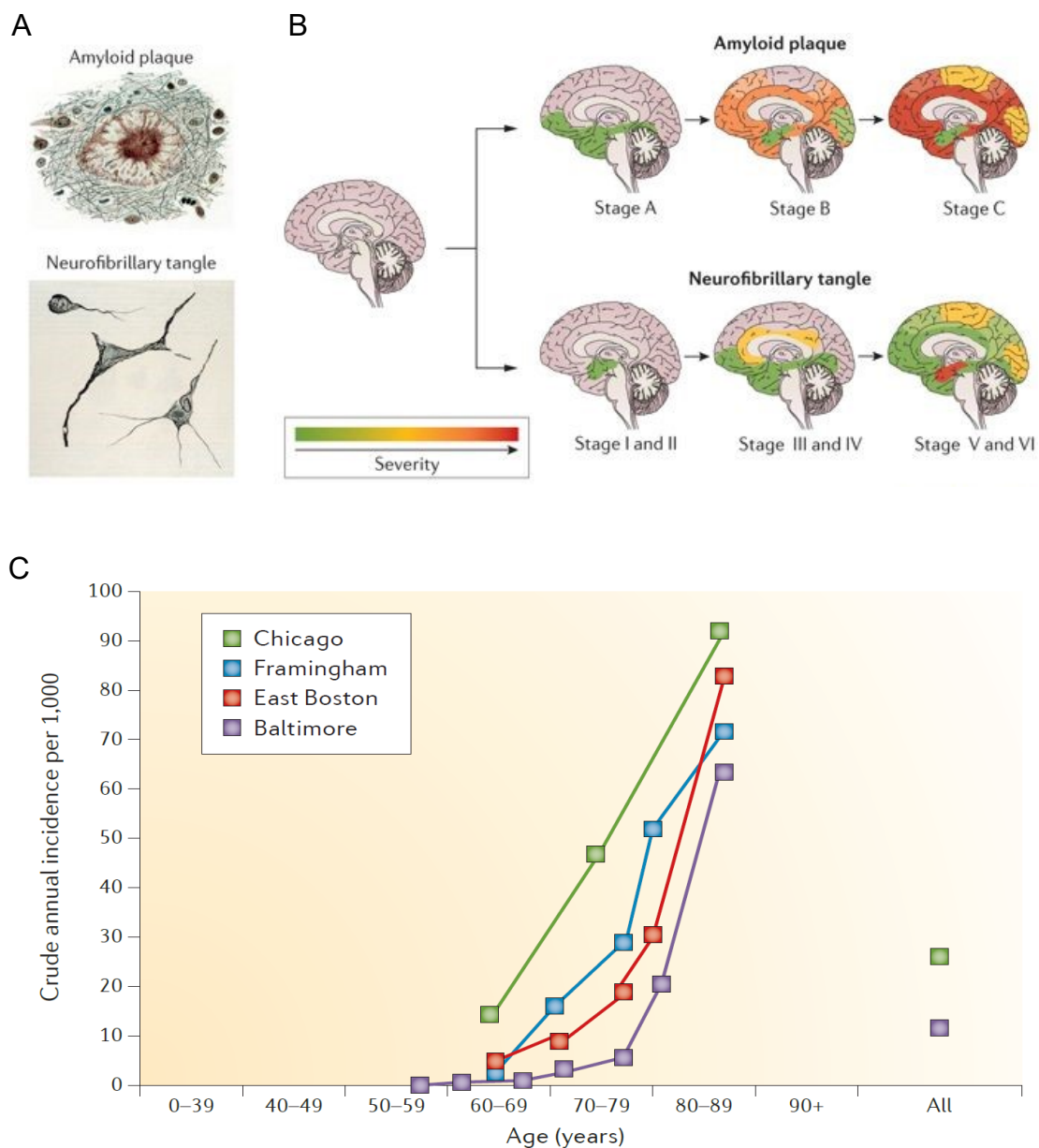


Figure 2: Clinical and pathological development of Alzheimer's disease.

A) A β plaques and NFT spread across the brain as the disease develops. Silver staining images envision the aggregated proteins, which are known to be the building blocks of extracellular A β plaques and intracellular NFT. B) In typical cases of AD, A β deposition precedes NFT and neuritic changes, originate in the frontal and temporal regions, hippocampus and limbic system. Occasionally, the disease seems to occur from other regions of the cerebral-neocortex (parietal and occipital lobes) rather than hippocampus. Stages of amyloid plaque deposition (stages A, B and C; front row) and NFT aggravation (stages I–VI; bottom row)). The NFT and neuritic deterioration starts in the medial temporal lobes and hippocampus and gradually spreads across the remaining areas of the neocortex. C) Comparison of data obtained from 4 large clinical trials (1, Framingham; 2, East Boston; 3, Chicago, and 4, Baltimore) shows that the incidence of AD rises exponentially after the sixth-decade of life. [Reproduced with permission from REF. (3), (Fig. 1&2) Nat Rev].

The amyloidogenic peptides are either rerouted to lysosomal compartments for degradation, or secreted into the extracellular space for removal. Any mutations in APP gene and/or presenilin (PSEN)1 or PSEN2 genes result in autosomal dominant, fully penetrant early-onset AD (4). Other mutations include the unfavourable apolipoprotein E (apoE) 4 allele which is strongly associated with increased brain A β deposition, majorly through impaired cellular A β uptake and clearance from the brain (15).

Several forms of extracellular and intracellular A β , such as monomeric, oligomeric and fibrillar forms, cause toxicity through several mechanisms including microglial infiltration, by generating reactive oxygen species (ROS), and/or synaptic damage (7). Besides genetic mutations in microtubule-associated protein tau (MAPT) gene, NFT can also be generated by A β -induced tau phosphorylation and cleavage; A β may also activate enzymes including GSK3 β , Cdk5, and multiple caspases, which trigger tau cleavage and phosphorylation. NFT-bearing neurons display loss of cytoskeletal microtubules and tubulin-associated proteins, leading to impaired microtubule assisted vesicular transport of nutrients across the cell, eventually leading to cell death (16, 17).

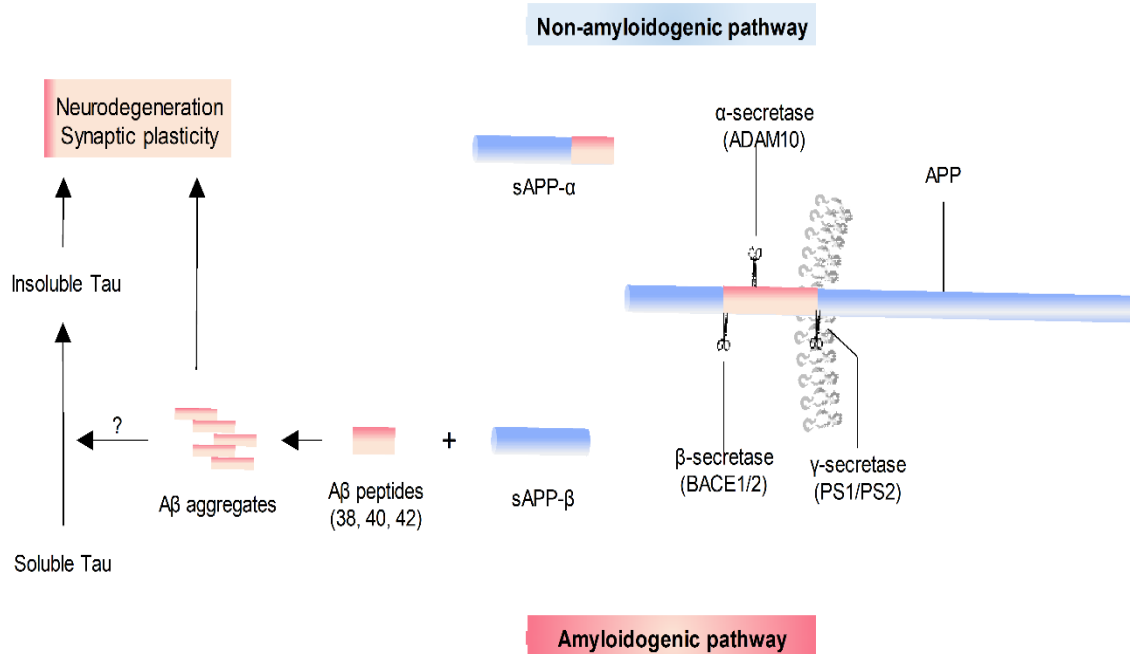


Figure 3: Mechanism of Amyloid-β genesis in a neuronal cell

Two predominant pathways namely the non-amyloidogenic pathway (ADAM10 mediated) and the amyloidogenic pathway (BACE 1/2 mediated) regulate APP processing. Sequential APP processing by β- and γ-secretases subsequently generate Aβ species which aggregate and may also trigger tau aggregation leading to neurotoxicity and neurodegeneration. Soluble (s)APP-α, soluble alpha-amyloid precursor protein; sAPP-β, soluble beta-amyloid precursor protein, ADAM10, A disintegrin and metalloproteinase domain-containing protein 10 or α-secretase; BACE1/2, beta-secretase 1/2; APP, amyloid precursor protein; Aβ, amyloid-beta peptides. [Adapted from Singh SK, *et al.* with the permission of Oxid Med Cell Longev (18)]

1.3 Amyloid-β catabolism in brain and periphery

An imbalance between production and clearance of Aβ is known to be the initiating factor in AD. Therefore, proper metabolism and maintenance of (low) Aβ levels is critical to maintain a healthy brain (19). Physiological Aβ metabolism occurs in central as well as peripheral compartments, communication between these two compartments occur primarily via blood-brain barrier (BBB). Predominant instances of AD are sporadic (~ 95%), manifested by the accumulation of Aβ, and mainly due to imbalances in Aβ production and clearance (14). Various pathways regulate Aβ degradation or its elimination from brain as shown in **Fig. 4**. Important pathways include cell-mediated phagocytosis, endocytosis, and macro-pinocytosis (mediated

by microglia, perivascular macrophages, astrocytes, oligodendrocytes and neuronal cells), enzymatic proteolytic degradation (mediated by neprilysin, insulin-degrading enzyme (IDE) and matrix metalloproteinases (MMP)), and A β transcytosis to the peripheral circulation primarily through the BBB (20), blood–CSF barrier mediated transport, interstitial fluid (ISF) bulk flow, as well as other pathways including arachnoid villi and glymphatic–lymphatic pathways (**Fig. 3**) (14). Endogenous inhibitors of A β aggregation such as the secreted ectodomain of tumour necrosis factor receptor superfamily member 16 and the N-terminal domain of myelin basic protein, avert A β deposition and enhance its efflux into the peripheral circulation (21, 22). It has been reported that ~60% of brain A β is cleared via transportation to the periphery (23, 24). Peripheral A β clearance pathways involve (i) A β uptake and phagocytosis by peripheral monocytes, macrophages, neutrophils, lymphocytes, and hepatocytes (25, 26); urinary or bile excretion (27, 28), (ii) degradation by proteolytic A β -degrading enzymes (29), and (iii) clearance from plasma mediated by A β -binding proteins and cells, such as erythrocytes, albumin, anti-thrombin III and lipoproteins, including apoE and apoJ/clusterin (30, 31). The central and peripheral pathways might interact with each other and work in synchrony to clear A β from the body (14).

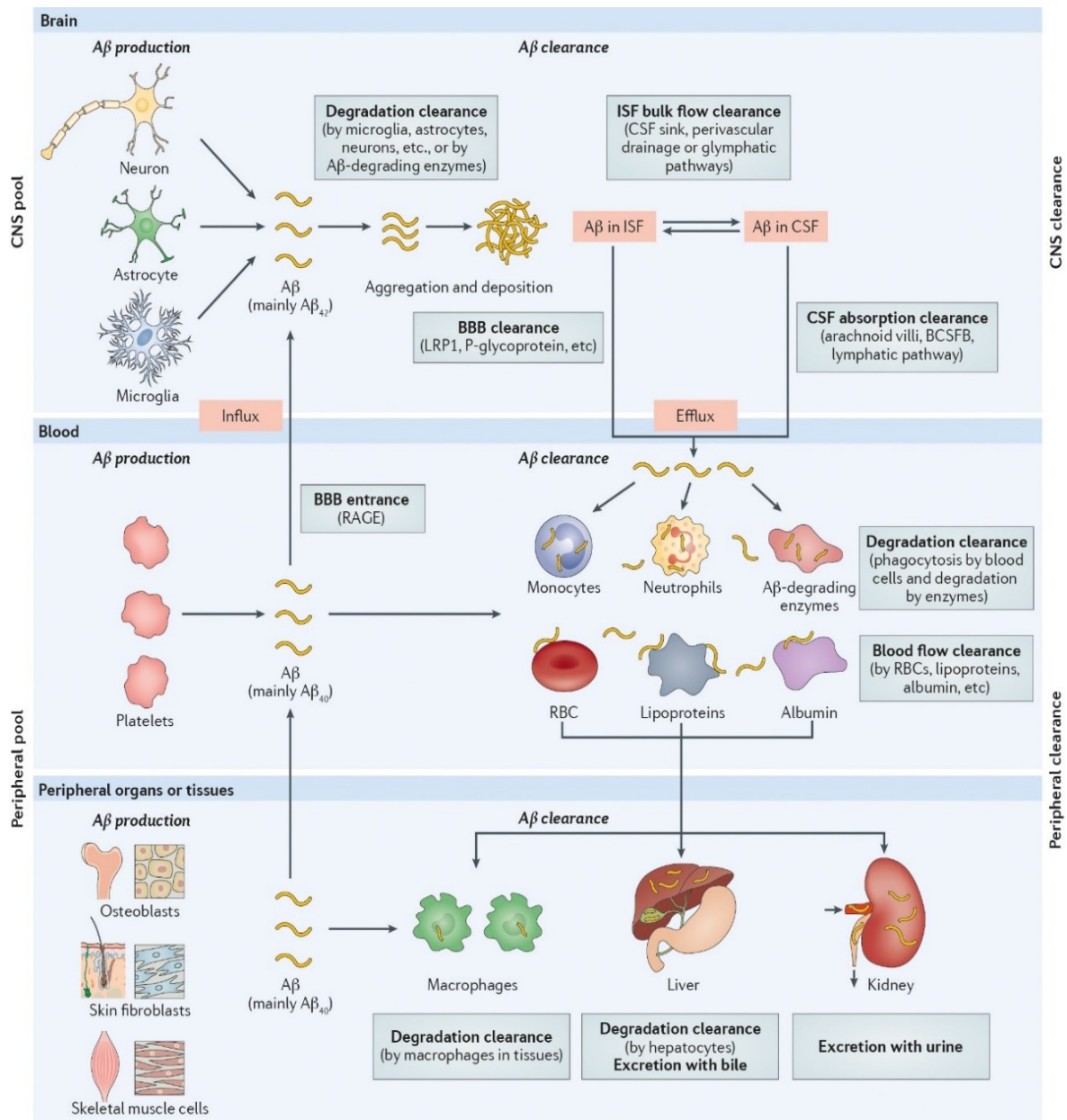


Figure 4: Amyloid-β clearance pathways

Amyloid-β (Aβ) is produced by various cells, both by central (neurons, microglia and astrocytes) and peripheral organs (blood platelets, skin fibroblast cells, osteoblast cells, and skeletal muscle cells). Partly, Aβ peptides in the CNS are metabolized/cleared via phagocytosis or proteolytic degradation, whereas others are transported into the blood for clearance via BBB, ISF bulk flow or cerebrospinal fluid (CSF) pathways. Aβ peptides in blood are phagocytosed, including by monocytes or neutrophils, some are degraded by Aβ-degrading enzymes, and some are transported by carriers (such as erythrocytes, albumin and lipoproteins) to peripheral organs or tissues, where Aβ peptides are degraded by macrophages or hepatocytes, or excreted via the liver or kidney. BCSFB, blood–CSF barrier; RAGE, receptor for advanced glycation end products; RBC, red blood cell. [Reproduced from Wang J, *et al.* with the permission of Nat Rev Neurol (14)]

1.4 Insulin production, function and pathways regulating insulin signaling

Insulin is a 5.8 KDa peptide hormone primarily secreted by the pancreatic β -cells in response to elevated blood glucose levels. Pancreatic β -cells synthesize insulin as pre-proinsulin, which is enzymatically processed to proinsulin and stored in secretory granules as microcrystalline arrays of zinc insulin hexamers (**Fig. 5**) (32). Glucose enters into the pancreatic β -cell via glucose transporters (GLUT) positioned on the plasma membrane. Once inside the cell, glucose is converted to pyruvate by the enzyme activity of glucokinase, eventually leading to adenosine triphosphate (ATP) production from pyruvate by the action of enzymes pyruvate dehydrogenase and carboxylase. Elevated cytoplasmic ATP/adenosine diphosphate levels cause closure of ATP-sensitive K^+ (KATP) channel, leading to membrane depolarization, in turn activating L-type voltage-dependent Ca^{2+} channels causing cell Ca^{2+} influx, and elevated cytosolic Ca^{2+} levels eventually leading to rapid insulin exocytosis from secretory granules into blood circulation (33, 34). Other major pathways regulating insulin release are vagus nerve stimulation of muscarinic receptors and β -adrenoceptor mediated insulin release via cyclic AMP (cAMP). Some minor pathways regulating insulin release from pancreatic β -cells include arginine mediated voltage-dependent Ca^{2+} channel activation and glucagon like peptide 1 (GLP-1) induced cAMP activation (35). Non-esterified fatty acids (NEFA) derived from dietary lipids or liver can increase hepatic glucose output and enhance insulin resistance by modulating glucose mediated insulin release from pancreatic β -cells.

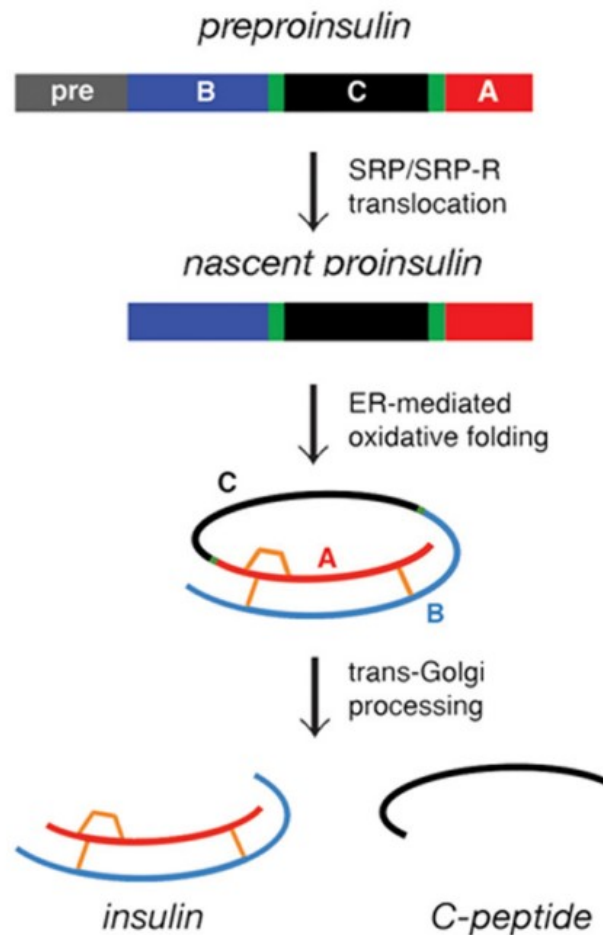


Figure 5: Biosynthesis of insulin from proinsulin and preproinsulin

Insulin is synthesized in pancreatic β -cells and it begins with preproinsulin, in the endoplasmic reticulum (ER) nascent preproinsulin undergoes disulfide pairing to generate proinsulin. Prohormone convertases, such as 'studies of protease' (i.e. SPC, 2&3), and carboxypeptidase E cleave proinsulin into insulin and C-peptide in the β -cell secretory granules. SRP & SRP-R, signal recognition particle and its receptor. [Reproduced from Yang Y, *et al.* with the permission of *J Biol Chem* (36)]

At cellular level, insulin plays a predominant role in carbohydrate, amino-acid and lipid metabolism and is involved in transcriptional regulation of mRNA (35). Major sites of insulin action include muscle, adipose tissue, liver, vascular endothelium, brain, pancreas, pituitary gland, kidneys, gonads and bone. In muscle tissue, insulin promotes glucose uptake by GLUT4 and enhances its utilization by converting glucose to glycogen by enzyme glycogen synthase. In adipose tissue, insulin promotes lipogenesis while repressing lipolysis, thereby maintaining circulating free NEFA levels (35). In liver, insulin promotes glycogen synthesis while modulating lipoprotein

metabolism and protein synthesis. Insulin plays a key role in endothelial function by enhancing nitric oxide (NO) production by modulating enzyme activity of NO synthase. In pituitary, insulin regulates the production of growth hormone. In kidney, insulin regulates gluconeogenesis and transport of micronutrients. In gonads, insulin is involved in steroidogenesis by enhancing estrogen and androgen production by granulosa and stromal cells respectively. In bone, insulin stimulates osteoblast and suppresses osteoclast activity thereby stimulating bone formation (35, 37).

In the CNS, insulin acts as a neuropeptide and a growth factor, and is known to modulate satiety, appetite, olfactory function, memory and cognition. Peripheral insulin is thought to be transported to brain across the BBB via saturable insulin receptor (IR) mediated uptake (38-42). Alternatively, under supraphysiological conditions, endothelial endosomal compartments take up peripheral insulin and transverse across the BBB as proposed in **Fig. 6.** (43). Insulin and its receptors are distributed throughout the CNS (more densely in the olfactory bulb, striatum, cerebral cortex and choroid plexus). Insulin regulates glucose uptake in the CNS via insulin sensitive GLUT4, whereas GLUT1 mediated glucose uptake at the BBB is independent of insulin. Neuronal specific insulin-mediated glucose uptake was observed in hippocampus and hypothalamus (44, 45). Insulin was shown to enhance hippocampal long-term potentiation (LTP) via N-methyl-D-aspartate (NMDA) receptor activation, LTP is a molecular measure for strong synaptic association between the neurons (46). Insulin was shown to have neuroprotective effects by activating insulin-signaling pathways which protect the neuronal cells and pericytes from oxidative stress and apoptosis (47, 48).

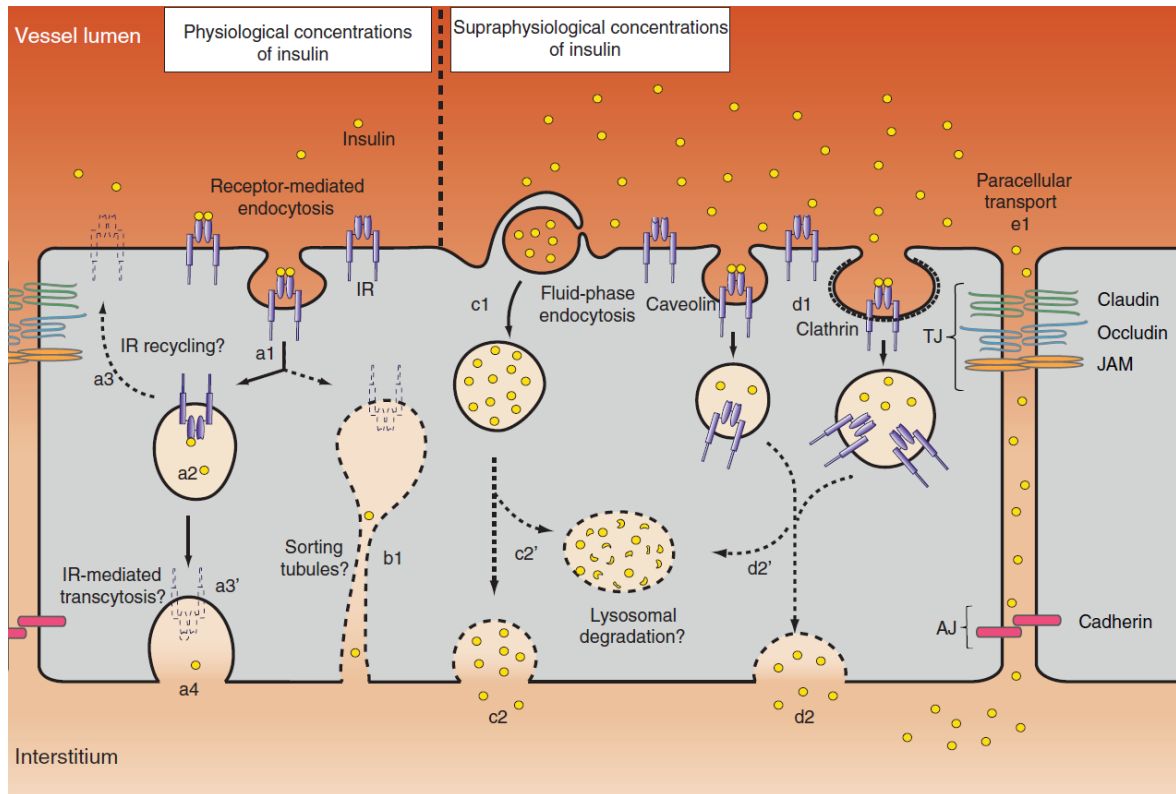


Figure 6: Possible pathways governing transcytosis of insulin across the endothelial barrier

Schematic depiction of potential transcellular pathways governing insulin transport across the endothelial barrier separating targeted tissue and the blood stream. Under physiological plasma concentrations, insulin is transported across the endothelium via receptor-mediated endocytosis. Inside the cell, receptors and insulin are sorted and the receptors are recycled back to the surface of the cell. Under supraphysiological conditions like hyperinsulinemic state, fluid-phase endocytotic vesicles, also helped by clathrin and caveolin, likely internalize insulin. Paracellular transport was also described to play a minor role in insulin trafficking across the endothelial barrier. [Reproduced from Yazdani S, *et al.* with the permission of *Traffic* (43)]

Insulin and their analogs signal through IR, a transmembrane tyrosine kinase receptor which is a heterotetramer made of 2 α and 2 β glycoprotein subunits connected by disulfide bonds, and is positioned in the cell membrane (49). Upon binding of insulin to α subunit, a conformational change enables ATP to bind to the intracellular component of the β subunit, which triggers β subunit phosphorylation conferring tyrosine kinase activity, which in turn leads to recruitment and phosphorylation of insulin receptor substrate-1 (IRS-1). IRS-1 acts as a scaffold to organize and facilitate signaling complexes. IRS proteins are recruited to the membrane receptors through both pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains

in their amino terminus and are subsequently phosphorylated by the activated receptors on multiple tyrosine residues that form binding sites for intracellular molecules that contain Src-homology 2 (SH2) domains. IRS-1 mediates two major signaling pathways termed phosphatidylinositol (3,4,5)-kinase (PI3K)/AKT and Grb2-SOS-Ras-MAPK pathways (50). Key pathways modulating insulin responses are shown in **Fig. 7**.

a. PI3K/ AKT pathway

Binding of PI3K SH2 domains to the regulatory subunits of tyrosine-phosphorylated IRS proteins activates PI3K, subsequently phosphorylating phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate the lipid second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP₃), which in turn activates AKT by phosphorylating serine and tyrosine residues, further leading to downstream signaling induction. PI3K regulates 3-phosphoinositide-dependent protein kinase 1 (PDK-1) activation, which in turn activates AGC protein kinases by phosphorylating serine/threonine residues (50, 51). AGC protein kinases include isoforms of AKT/protein kinase B (PKB), p70 ribosomal S6 kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK), as well as several isoforms of protein kinase C (PKC). AKT phosphorylation at Ser-473 is prerequisite for full activation, which is accomplished by the mammalian target of rapamycin complex 2 (mTORC2) (52, 53). Three isoforms of serine/threonine protein kinases of AKT/PKB family were reported (54). PH domain allows AKT interaction with PIP₃ and its recruitment to the plasma membrane. Out of these three isoforms, AKT2 plays a predominant role in mediating insulin-mediated metabolism, and it is primarily disturbances in AKT2 that lead to insulin resistance under diabetic conditions (50).

Mammalian target of rapamycin complex 2 and PDK-1 mediated activation of AKT further phosphorylate and activate downstream targets. One such example is phosphorylation of tuberous sclerosis complex protein 2 (TSC-2) which in turn induces degradation of the tumor suppressor complexes TSC-2 and TSC-1, thereby activating mTORC1 complex. Activated mTORC1 initiates a cascade of downstream events such as phosphorylation and inhibition of 4E-binding protein 1 (4E-BP1), activation of ribosomal protein S6 kinases and sterol regulatory element-binding pro-

tein-1 (SREBP-1). and initiates regulation of a network of genes directing metabolism, protein synthesis, and cell growth (55). Transcription factors of the Forkhead box O (FOXO) family regulate lipogenic and gluconeogenic genes. AKT mediates exclusion of FOXO from the nucleus and blocks its transcriptional activity (55).

b. The Grb2-SOS-Ras-MAPK pathway

This pathway is activated independently of PI3K/AKT. Triggered insulin /insulin-like growth factor 1 (IGF-1) receptor and IRS proteins possess docking sites for adaptor molecules containing SH2 domains such as Grb2 and Shc (50). The carboxy-terminal SH3 domain of Grb2 binds to proteins such as Gab-1, whereas the amino-terminal SH3 domain binds to proline-rich regions of proteins such as son-of-sevenless (SOS), a guanine nucleotide exchange factor (GEF) for Ras. SOS catalyzes the switch of membrane-bound Ras from an inactive, GDP-bound form (Ras-GDP) to an active, GTP-bound form (Ras-GTP) (50). Ras-GTP then interacts and stimulates Ser/Thr kinase Raf, which instigates its downstream targets MEK1 and 2 that phosphorylate and activate the mitogen-activated protein (MAP)kinases ERK1 and 2 (50). Stimulated ERK1/2 play a vital role in key cellular events like cell proliferation or differentiation, regulating gene expression or extra-nuclear events, such as cytoskeletal reorganization, through activation of targets in the cytosol and nucleus (50).

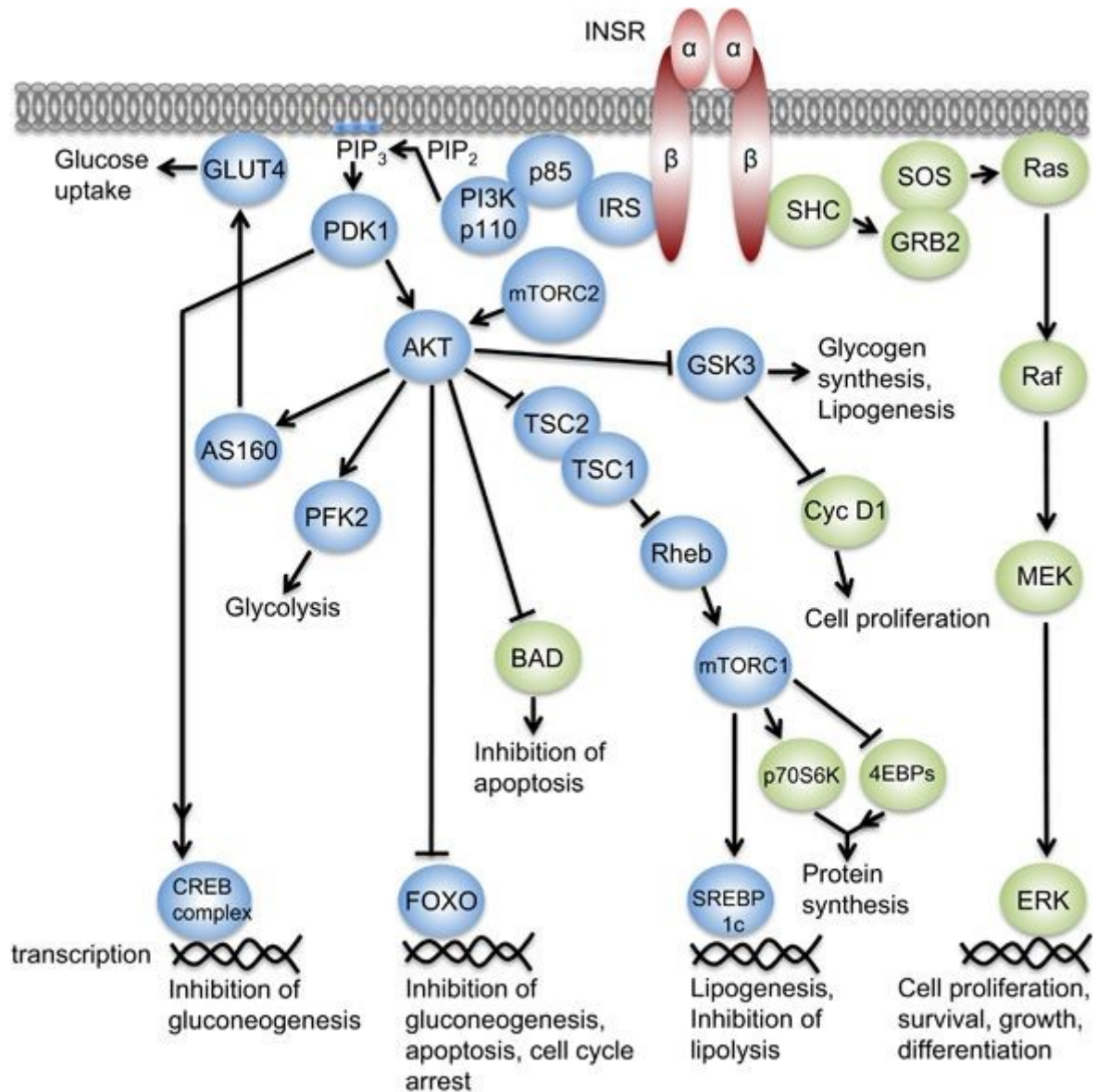


Figure 7: Pathways governing insulin signaling

Activation of IR initiates a cascade of phosphorylation events leading to a conformational change and autophosphorylation of the receptors, further leading to the recruitment and phosphorylation of receptor substrates such as IRS and Shc proteins. IRS activates the PI3K-AKT pathway by recruiting and activating PI3K, leading to the generation of second messenger PIP₃, whereas Shc activates the Ras-MAPK pathway. Membrane-bound PIP₃ recruits and activates PDK-1 thereby phosphorylating and activating AKT and atypical PKCs. AKT mediates most of insulin's metabolic effects, regulating glucose transport, lipid synthesis, gluconeogenesis, glycogen synthesis, cell cycle and survival. The Shc-Grb2-SOS-Ras-Raf-MAPK pathway controls cell proliferation and gene transcription. [Reproduced from Poloz Y, *et al.* with the permission of Cell Death Dis (51)].

1.5 Type-2 diabetes mellitus, a risk factor for Alzheimer's disease?

A large body of epidemiological evidence suggests that pre-diabetic insulin resistance, obesity, and T2DM are risk factors for memory related disorders such as AD (51, 56-71). Similarly, insulin resistance has been linked to progression of neurodegenerative diseases by promoting disease-specific pathological lesions, thereby elevating the risk of neuronal vulnerability leading to neurodegeneration (72, 73). The outcome of various studies using T2D animal models strengthens the concept that T2DM promotes the development of AD pathology, such as A β plaques, tau phosphorylation and neurofibrillary lesions, and α -synuclein lesions (**Fig. 8**) (74, 75). Neuroimaging studies identified changed brain patterns in T2DM which were similar to those observed in other neurodegenerative dementias, including white matter lesions (76). Volumetric MRI studies identified a reduction in hippocampal volume in T2DM and obese individuals (77-87). Furthermore, changes in cerebral blood flow and oxygenation have been detected in T2DM individuals (88-93). Longitudinal clinical studies showed that in elderly diabetic human subjects insulin resistance strongly associates with AD, and diabetic apoE 4 carriers are prone to elevated risk of AD with cerebrovascular amyloid deposits (94-97). In contrast, a post-mortem immunohistochemical study found no association between T2DM and the severity of AD pathology in brain in humans (98). Indeed, a couple of clinical studies reported no correlation between measures of longitudinal glucose intolerance and post-mortem AD pathology (73, 99), or no significant alterations in brain A β load between non-demented elderly subjects with or without T2DM (100). Similarly, no differences in PET-assessed A β load was observed in a large sample size of diabetic versus nondiabetic elderly individuals, neither with normal cognition, nor with MCI or AD (87). Another PET study also reported no quantitative differences in brain A β levels between individuals with clinical AD with or without diabetes mellitus (101). Surprisingly however, low frequency of A β -positive positron emission tomography scans were detected in a study on T2DM patients who had been clinically diagnosed with AD (102). Most of the clinical studies were cross-sectional and compared individuals with late clinical AD symptoms, which fail to take into account the time course of disease progression in AD since A β deposition in the brains of individuals with AD commences 10–20 years before the appearance of clinical symptoms (103).

On the other hand, AD and T2DM are characterized by many pathophysiological similarities like high prevalence with aging, genetic predisposition and similar pathological features in brain and pancreatic islets. Growing evidence links precursors of amyloid deposition in the brain and pancreas with the pathogenesis of AD and T2DM, respectively (104). T2DM can be a potential risk for mild cognitive impairment (MCI) developing into AD, by prompting vascular dysfunction and oxidative stress (105). A Southeast Asian longitudinal study reported clear evidence that T2DM was associated with augmented incidence of MCI and its advancement to dementia (106). Coming back to the murine studies, Chauhan and colleagues reported AD like pathology and memory deficits when T2DM was induced in aging-accelerated mice, and animals exhibited elevated A β and hyperphosphorylated tau protein levels (107). Similarly, AD transgenic mice subjected to a high-fat diet displayed elevated A β and tau levels in conjunction with activated astrocytes in mice brain (108). In contrast, caloric restriction has been shown to decrease A β deposition in both elderly mice (109) and transgenic mouse models of AD (110). It was hypothesized that decline in IGF-1 and abrogated peripheral insulin responses hinder uptake of IGF-1 and insulin into the brain, resulting in impaired insulin responses, which regulate A β metabolism in brain (111, 112).

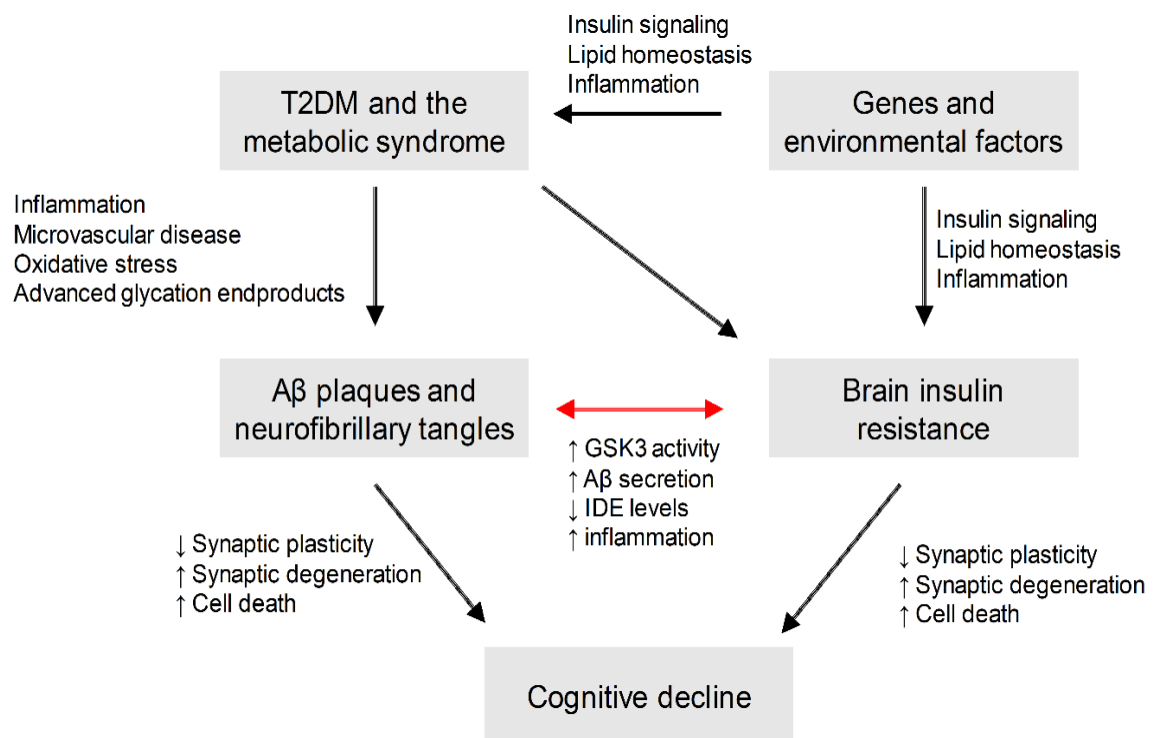


Figure 8: Correlation between T2DM, brain insulin resistance, and AD

A vast number of diverse studies carried out in animals, epidemiological and clinical studies derive a crucial nexus between metabolic disease and AD. Lifestyle and genetic factors manifest early changes in insulin resistance in central and peripheral tissue. These changes may lead to T2DM and metabolic syndrome, which may further promote changes in brain insulin signaling or resistance as well as promote AD pathology through hyperinsulinemia, inflammatory stress, ROS, and AGEs. Furthermore, AD pathology manifested by A β burden and impaired brain insulin sensitivity or resistance may drive one another in a positive feedback loop, which eventually leads to synaptic degeneration and symptoms of clinical dementia manifested in AD. Arrows in black indicate the association between various pathological states, whereas red arrows indicate the feedback loop between A β production and brain insulin resistance. [Adapted from Yarchoan M, *et al.* with the permission of *Diabetes* (113)].

1.6 Brain insulin signaling in Alzheimer's disease

Changes in (early) brain insulin signaling and IR levels in AD individuals were observed across multiple studies, and the reasons behind that and its influence on A β metabolism and memory in AD has been a topic of debate (114-120). In a randomized clinical study, elderly individuals were identified with low brain insulin levels and decreased insulin affinity towards IR when compared with young and middle-aged healthy individuals without dementia (121). In a longitudinal aging study, higher insulin binding with IR was observed in young and middle aged individuals compared to elderly AD individuals. Surprisingly, insulin binding to IR was higher in the AD group compared with non-demented subjects (120, 122). Subsequent studies reported reduced brain IR mRNA expression and protein levels, and reduced insulin binding to IR in AD individuals compared with age matched controls (117, 120). However, some studies identified no such changes in IR levels and their association with AD (123). For the first time in 1985, Hoyer reported central insulin resistance and dysfunctional insulin signaling in late onset AD (LOAD) (121, 124, 125). Later, the Rotterdam study confirmed a strong association between T2DM and LOAD (65). Subsequently, successive clinical and epidemiological studies confirmed a potential association, representing altered metabolic functions and parameters such as hyperglycemia and hyperinsulinemia correlates positively with the development of LOAD, mainly with the cognitive impairment (117, 126-130). Frölich and colleagues reported significantly reduced IR levels in cerebral cortex and hippocampus in LOAD patients (121). Later, de la Monte confirmed reduction in insulin

and IGF-I receptor levels in frontal cortex, hippocampus, and hypothalamus of AD patients (120).

Insulin is known to alter A β metabolism and promote sAPP α secretion in a concentration- and tyrosine kinase-dependent manner (131, 132). Similarly, insulin and IGF-1 protect neurons from A β -induced neurotoxicity in neuronal cultures by modulating A β transport and/or production at the blood–brain interface (133). Importantly, A β and insulin share common pathway of degradation mediated by insulin degrading enzyme (IDE) and regulated by insulin mediated PI3K activation. Under physiological conditions, insulin and IR interaction generates a cascade of events which regulate key downstream serine/threonine kinases such as AKT, mTOR and ERK. In neuronal cells PI3K, AKT, GSK3, BCL-2 agonist of cell death (BAD), FOXO, mTOR and MAPK pathways are regulated by IR activity. Any alteration in physiological activity of these pathways might abrogate neuronal function, supporting the hypothesis that insulin resistance/impaired insulin signaling and/or function could promote AD (134).

Both A β and insulin are amyloidogenic peptides, and share common motifs and similar binding domains. A β is known to compete with insulin for IR and can interfere with insulin-mediated responses in brain (135, 136). Correspondingly, a strong interaction with IR and a receptor conformational change with amyloidogenic A β isoforms were reported by Martins and colleagues (135). On the other hand, the presence of A β oligomers led to substantial loss of neuronal IR specifically on the surface of dendrites inducing rapid internalization and redistribution of IR in their cell bodies which lead to impaired responsiveness to insulin (137, 138). Since IR plays a predominant role in regulating key neurological processes such as memory and tau phosphorylation, A β -induced loss of membrane IR might represent a significant early modification underlying memory impairment and aggravation of AD. Furthermore, A β oligomers augment proinflammatory TNF- α levels, in turn activating c-Jun N-terminal kinase (JNK) in neurons, ultimately leading to IRS-1 serine phosphorylation (IRS-1pSer) and blocking downstream insulin signaling activation (**Fig. 9**) (114, 139).

Endorsed by these findings, increasing brain insulin levels to modify AD profoundly gained interest among the scientific community. Randomized clinical studies

using intra-nasal administration of insulin in patients with AD and MCI improved memory and recall (140). Similarly, a subsequent trial in MCI and AD individuals was reported in which intra-nasal insulin therapy improved cognitive and functional capacities as well as improved fluorodeoxyglucose F18 - positron emission tomography (FDG-PET) metabolism, an indicator of glucose uptake and metabolism in brain. Insulin sensitizing therapeutics gained interest as potential disease modifying therapeutics in AD (141). Thiazolidinedione-derived nuclear peroxisome proliferator-activated receptor- γ (PPAR γ) agonists, metformin, rosiglitazone and glucagon-like peptide 1 (GLP-1) targeting drugs showed some improvements in human and mouse, MCI and AD models (142).

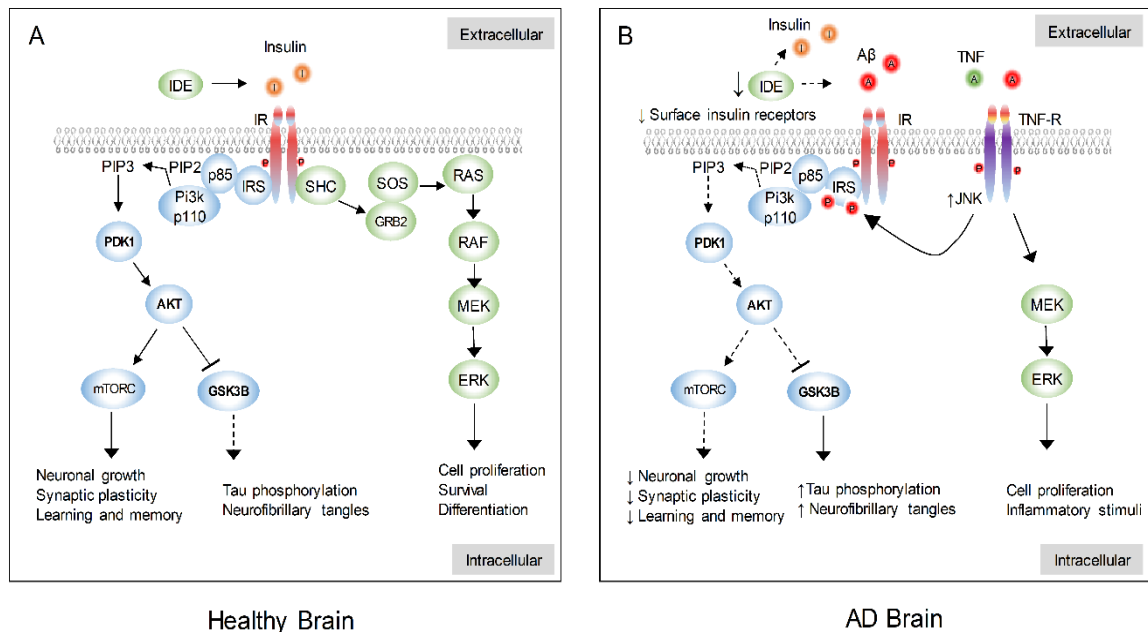


Figure 9: Distinctions in brain insulin signaling in healthy and AD subjects

Scheme representing possible pathways of neuronal insulin signaling in (A) normal and (B) AD brain. Under physiological conditions, insulin triggers IR mediated phosphorylation of insulin receptor substrate-1 (IRS-1), in turn activating phosphoinositide 3-kinase (PI3K) and downstream cellular responses which facilitate neuronal growth, memory, and/or a balance in vasoconstriction and vasodilation by modulating eNOS activity. In AD, amyloid- β (A β) accumulation induces activation of tumor necrosis factor- α (TNF- α) receptor and stress kinases such as c-Jun N-terminal kinase (JNK) resulting in inhibitory serine phosphorylation of IRS-1. Insulin resistance appears to effect the expression of A β -degrading insulin degrading enzyme (IDE). Lowered IDE levels further affect A β degradation. Impaired brain insulin signaling increases GSK-3 β activity, leading to abnormal tau phos-

phorylation. Deficient insulin signaling leads to impairment in nerve growth, synaptic plasticity, learning and memory. Aberrant phosphorylation of IRS causes an imbalance in homeostatic regulation of vascular function. Decreased production of NO may result in decreased cerebral blood flow and increased release of pro-inflammatory cytokines and reactive oxygen species production [Adapted from Bedse G, *et al.* with the permission of Front Neurosci (143)].

1.7 Association between brain insulin receptor- β and low-density lipoprotein related protein-1

Low-density lipoprotein related protein-1 (LRP-1), also known as cluster of differentiation (CD) 91 or α 2-macroglobulin receptor (α 2MR), is a 85 kDa type 1 transmembrane protein which belongs to the LDL receptor family and is implicated in protease homeostasis and lipoprotein metabolism (144, 145). LRP-1 shares structural and functional similarity with other LDLR family members, which include LDLR, LRP subtypes 1, 1B, 5 and 6, megalin/LRP2, VLDL receptor, apoE receptor 2, and sortilin-related receptor (SorLA), all having distinct physiological roles in cellular trafficking and/or signaling, based on their localization and tissue-specific expression (26, 146). LRP-1 is generated upon cleavage of the ~ 600 kDa precursor protein by furin in the golgi apparatus, generating a 515 kDa α chain and 85 kDa membrane-bound β chain (147, 148), together, both the α and β chains of LRP-1 are known to bind >40 ligands that are involved in numerous cellular processes (149, 150).

LRP-1 has two major known functions: 1) it acts as a scavenger receptor participating in the endocytosis of various ligands; 2) it functions as signaling receptor and binding partner for various receptors with enzyme activity, thereby modulating various cellular events and/or responses (144, 148, 151). Ligand binding to LRP-1 promotes internalization through endocytosis and allocates to endosomal/lysosomal compartments. Upon dissociation of a ligand, LRP-1 gets recycled back by interacting with sorting nexin 17 (152). Primarily motifs conjugated to the cytoplasmic tail, namely YXXL, dileucine, and NPXY are responsible for highly effective and controlled endocytosis and recycling (153). LRP-1 mediates cell signaling pathways by three diverse molecular mechanisms: i) by functioning as an endocytic receptor for signaling complexes, for example with APP, urokinase receptor, platelet derived growth factor receptor, α 2M, apoE, and matrix metalloproteinases known to activate cytoplasmic signaling pathways in an LRP-1 dependent fashion (154-156); ii) by

associating with ligands, for example tPA mediated Src kinase activation or activation of AKT and ERK kinases (157); iii) gamma-secretase processed LRP-1 can translocate to the nucleus and regulate signaling and nuclear transcriptional regulation of genes (158).

Bu and colleagues identified that LRP-1 is a key binding partner of the IR- β subunit and is able to regulate insulin signaling and glucose uptake in neuronal cells (**Fig. 10**). Accordingly, LRP-1 deficient mice demonstrated loss in insulin signaling as well as reduced levels of glucose transport molecules, primarily glucose transporters GLUT3 and GLUT4, leading to reduced insulin mediated glucose uptake (159). LRP-1 knock-down in neuronal cells and in mouse brain impaired IR- β levels, thereby hindering insulin mediated activation of AKT and downstream signaling (160). Neuronal LRP-1 deficient mice develop global deficits in brain lipid metabolism further leading to progressive age-dependent dendritic spine deterioration, loss in synaptic function, inflammation, and eventual neurodegeneration (161).

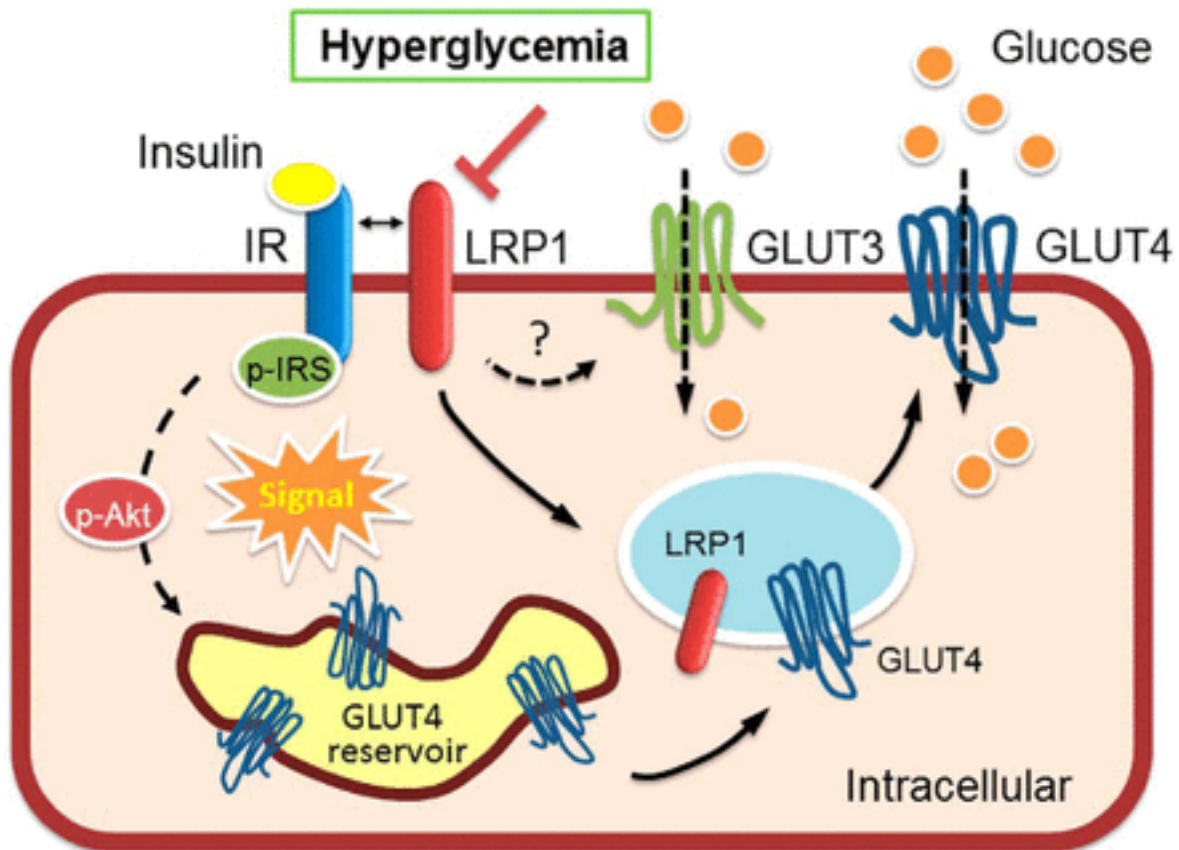


Figure 10: Prototype of LRP-1 regulating insulin signaling and glucose metabolism in neuronal cells

Interaction of LRP-1 and IR interaction and subsequent stimulation of insulin signaling. LRP-1 mediates glucose metabolism by modulating surface expression and function of GLUT4. Hyperglycemia deters LRP-1 levels which may further impair LRP-1 and IR interaction necessary for insulin signaling and glucose homeostasis in the brain. [Reproduced from Liu CC et al. with the permission of J Neurosci (159)].

1.8 The blood-brain barrier and its function

The microvasculature distributed in the CNS, termed as the BBB, possesses unique properties. Brain microvasculature is made-up of continuous and non-fenestrated brain capillary endothelial cells (BCEC) which reside over a basement membrane and are provided with tight junctions. BCEC are surrounded by pericytes and astrocyte end-feet and tightly regulate uptake and transport of micro and macronutrients to brain (**Fig. 11**). Several specific proteins form the unique tight junctions by sealing the gaps and holding the endothelial cells together, thereby protect the CNS from invasion of pathogens, toxins and xenobiotics (162).

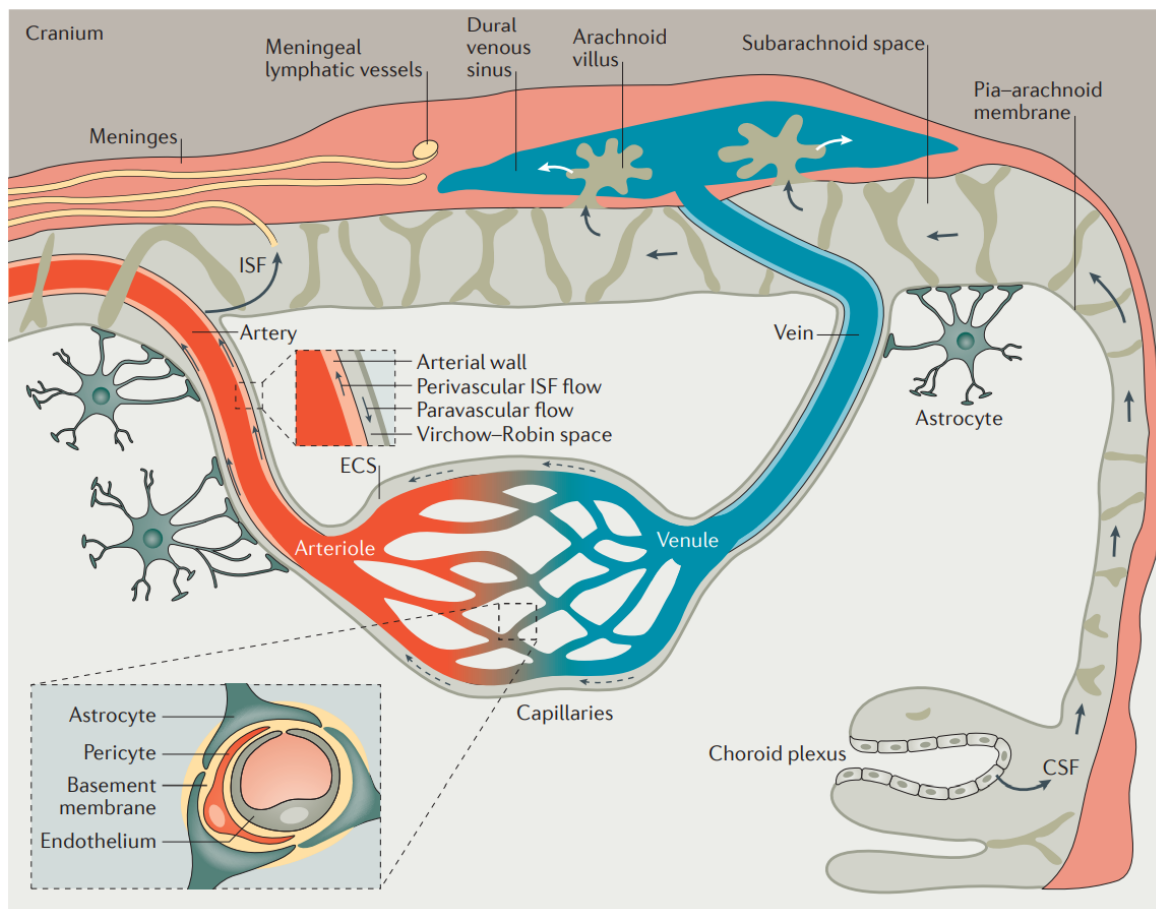


Figure 11: Structure of the BBB

Brain capillaries constitute the BBB. The cross section of BBB (large inset) shows tight endothelium resting on the basement membrane with pericytes and extensions of astrocyte filaments known as astrocyte end-feet wrapping around the capillary structure. Cross section of the artery (small inset) shows the flow of ISF, which is opposite to arterial blood flow. CSF is produced in the choroid plexus, which flows from brain ventricles into subarachnoid spaces, further draining into the meningeal lymphatic system and/or venous blood through the arachnoid villi. [Reproduced from Sweeney, M. D. *et al.* with the permission from Nat Rev Neurol (163)]

Water-soluble micronutrients like ions, amino acids, proteins and vitamins readily cross the BBB using carrier mediated transport systems. However, BBB transcytosis of larger molecules like insulin, leptin and iron are mediated through receptor mediated transcytosis. The BBB harbors various receptors which mediate receptor mediated endocytosis upon ligand binding, and are mostly associated with clathrin and/or caveolin dependent endocytotic pathways, for example IR, IGF-1R,

transferrin receptor, LRP-1 and 2, diphtheria toxin receptor, and RAGE (162, 164). Forming the critical interface between the blood and brain, the BBB is supposedly also responsible for IR mediated insulin uptake into the brain (42), whereas, LRP-1 and megalin receptors mediate A β clearance from the brain (165). The key functions and properties of the BBB are shown in **Fig. 12**. Loss in function and BBB integrity was identified in post-mortem brain tissue from AD subjects, identified by accumulation of blood derived macromolecules like fibrinogen, thrombin, albumin and IgG in the prefrontal, entorhinal and hippocampal regions in brain (163, 166-171).

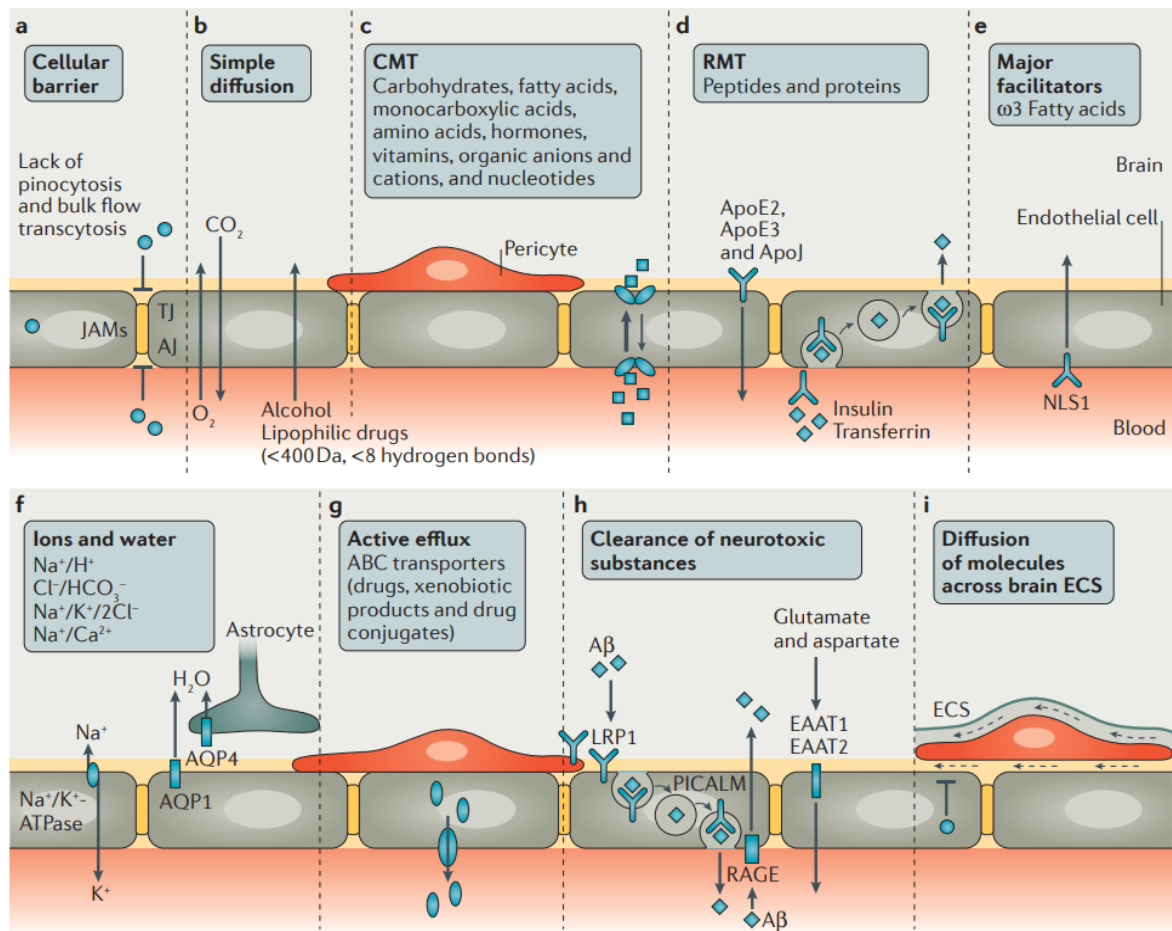


Figure 12: Diagram showing key transport properties of the BBB

a) At the BBB endothelium, tight junction (TJ) formations, adherent junctions (AJ) and junctional adhesion molecules (JAM) strictly regulate paracellular exchange of metabolites. The distinct ability to restrict pinocytosis and bulk flow-mediated transcytosis contribute to the BBB function. b) Exchange of gasses such as CO₂ and O₂, and small lipophilic compounds are transported across the endothelium via simple diffusion. c) Large metabolites and macronutrients (carbohydrates, fatty acids, amino acids, vitamins, organic anions and/or cations and nucleotides) use carrier mediated transport (CMT) to cross the BBB, based on specificity of the substrate and its concentration gradient.

d) Peptides and proteins (i.e., apoE2/3, apoJ/clusterin, insulin and transferrin) use receptor mediated transport (RMT) to cross the BBB. e) Facilitatory transport mediates transcytosis of ω 3 fatty acids to brain via sodium-dependent lysophosphatidylcholine symporter 1 (NLS1). f) Electrolytes and ions are transported across the BBB using ATP dependent transporters like abluminal sodium-potassium ATPase pump (Na^+/K^+ -ATPase), luminal sodium-hydrogen symport pump (Na^+/H^+), abluminal chloride-bicarbonate pump ($\text{Cl}^-/\text{HCO}_3^-$), luminal sodium-potassium-chloride symporter ($\text{Na}^+/\text{K}^+/\text{Cl}^-$) and luminal sodium-calcium ($\text{Na}^+/\text{Ca}^{2+}$) exchange pump. Aquaporin (AQP) receptors: AQP1 (on endothelial cells) and AQP4 (on astrocyte end-feet) drive water transport across the BBB. g) ATP-binding cassette (ABC) active efflux transporters on the luminal and abluminal surface of the BBB regulate the entry of xenobiotic into the brain. h) Neurotoxic compounds like $\text{A}\beta$ peptides are removed from the brain via phosphatidylinositol-binding clathrin assembly protein (PICALM/CD31)-mediated transcytosis and through LRP1 present on the abluminal surface of the BBB. Whereas, $\text{A}\beta$ influx from blood to brain is carried out by the RAGE located on the apical side of the BBB, can contribute to $\text{A}\beta$ accumulation and inflammation in brain. Toxic buildup of glutamate and aspartate in the brain is transported back to peripheral circulation across the BBB via CMT transporters, excitatory amino acid transporters 1 & 2 (EAAT1 & 2). i) Solute diffusion in the brain extracellular spaces (ECS) (dotted arrows) are removed from the brain via transvascular transport. [Reproduced from Sweeney, M. D. *et al.* with the permission from Nat Rev Neurol (163)]

1.9 Amyloid- β clearance pathways at the BBB

Unbound, soluble $\text{A}\beta$ peptides can be transported across the BBB via receptor mediated pathways (172). Similarly, $\text{A}\beta$ can bind to carrier proteins such as albumin, apoE2/E3, apoJ/clusterin, transthyretin, and α 2M to form complexes (173) and sequester in ISF or CSF in the brain which may facilitate receptor mediated transport across the BBB. Unbound $\text{A}\beta$ tends to form insoluble fibrils and oligomers causing synaptic loss and neurotoxicity. In AD, due to imbalances in $\text{A}\beta$ production versus clearance from the brain, $\text{A}\beta$ gets trapped at the basement membrane of BCEC causing cerebral amyloid angiopathy (CAA) which leads to functional and physiological alteration of the BBB. This is often accompanied by cerebral microbleeds or lobar intracerebral hemorrhage (174). Among thus far identified genetic risk factors, dominant apoE 4 and 2 alleles are associated with the highest risk of sporadic CAA (175).

Under pathological conditions like AD, the distribution of influx and efflux transporters at the BBB modifies or loses their chaperone properties, aggravating the AD pathology (19). A handful of receptors were identified to clear $\text{A}\beta$ from the

brain. These include LRP-1, ABCB1/P-glycoprotein (Pgp) and ABCC1/multidrug resistance protein 1 (MDR1) (176-178). Similarly, RAGE receptor is known to take up A β peptides by BBB and reroute it into brain parenchyma (167). Acknowledging the BBB role in regulating A β uptake and clearance, its dysfunction under certain pathological conditions is the major factor leading to vascular dementia and CAA (165). Key pathways regulating A β trafficking at the BBB were shown in the **Fig.13**.

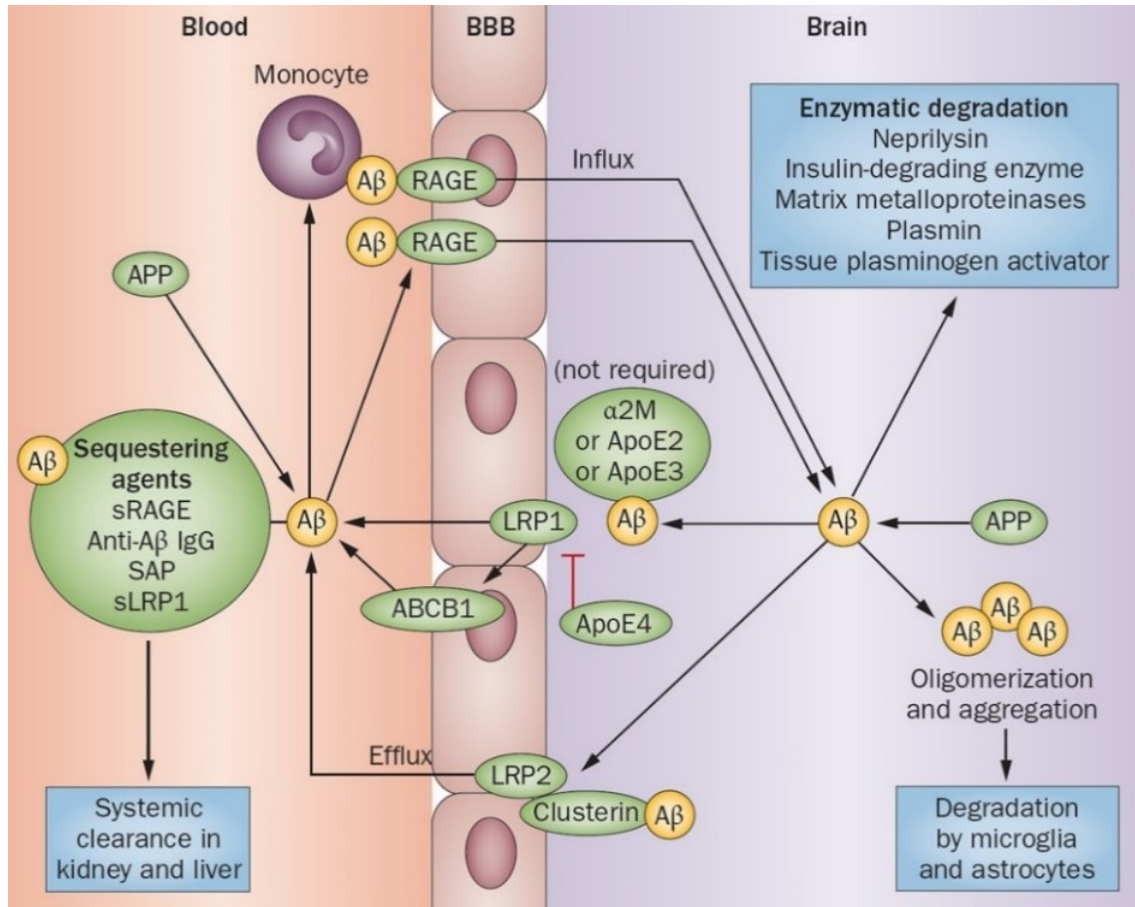


Figure 13: Pictorial representation of A β clearance pathways at the BBB

The BBB restricts systemic environment to enter the CNS, and mediates receptor mediated trafficking of A β transport, majorly mediated by LRP-1 (efflux from the brain) and RAGE (uptake into the brain). Chaperon proteins namely apo E2/E3, Apo J, transthyretin, and α 2M facilitate receptor mediated endocytosis and/or clearance from brain via LRP-1 and LRP-2 receptors. ADE: amyloid- β degradation enzyme, (s)LRP: soluble low-density lipoprotein receptor-related protein, (s)RAGE: soluble receptor for advanced glycation end products, α 2M: α 2-macroglobulin, SAP: serum amyloid P [Reproduced from Tarasoff-Conway JM, *et al.* with the permission from Nat Rev Neurol (20)].

1.10 Pathways regulating intracellular A β degradation and protein homeostasis

Two major pathways regulating cell homeostasis of A β peptide levels are the ubiquitin-proteasome system (UPS) and autophagy-lysosomal processing (ALP) (**Fig. 14**). Both systems maintain cell homeostasis by digesting misfolded and faulty proteins, which is critical for cell survival (179-181). Various studies identified a perturbed or weakened proteasome-mediated degradation system in AD. Moreover, A β peptides were shown to bind to the proteasome inner catalytic ring thereby protecting ubiquitinated proteasome substrates from degradation (182-184). On the other hand, inhibition of the proteasome prevents PSEN1&2 degradation and can accelerate APP maturation, enhancing A β production in HEK293 cells (184, 185). Even though there are evidences that support an impaired proteasome system in AD, its functional role in progression of AD is still elusive (186).

Predominantly cell surface proteins are internalized through receptor-mediated endocytosis and degraded by cathepsins (B, H, L & D) and hydrolases (like phosphatases, nucleases, proteases, and glycosidases), within the lysosomes (187-189). More importantly, proteins involved in A β production such as APP, A β , and apoE are processed via endosome-lysosomal degradation pathway (190, 191). It has been reported that endosomes and lysosomal complexes colocalize in the proximity of γ -secretase complex, APP and CTFs (189). Some studies suggest that lysosomal A β accumulation in AD brain may be one of the consequences of impairment in lysosomal efficiency. Moreover, autophagy disruption lead to neurodegeneration and further promoted amyloidosis in the earlier stages of AD, and in brain regions vulnerable to amyloidogenesis like hippocampus and entorhinal cortex, which react by increasing lysosomal biogenesis and activity (192). Aging resulted in increased lysosomal expression and localization of aspartyl proteases in cortical and brainstem neurons, which may suggest altered APP metabolism (189, 190, 193).

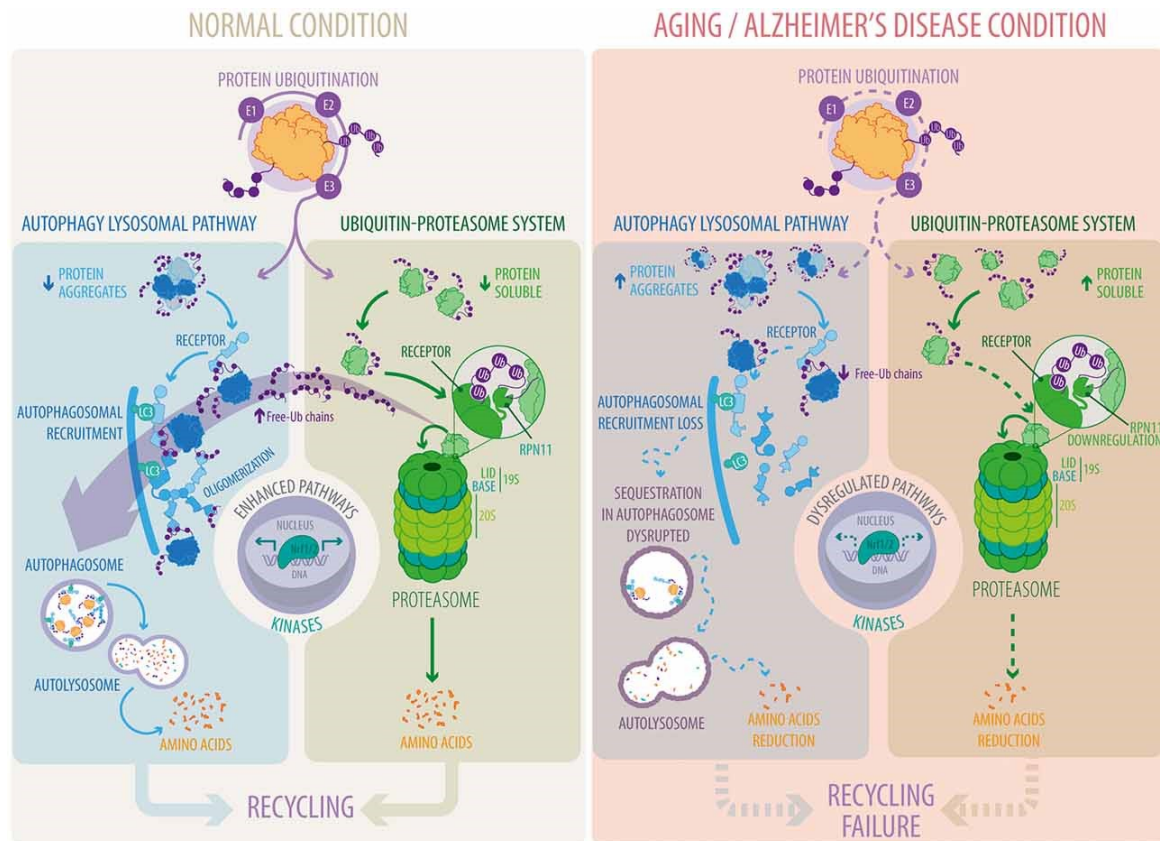


Figure 14: Protein degradation pathways (autophagy-lysosomal processing and ubiquitin proteasome system) under healthy and AD conditions

Pictorial representation of potential autophagy-lysosomal processing (ALP) and ubiquitin proteasome system (UPS) regulation in healthy and diseased states. Under healthy state, ubiquitinated substrates are recognized by ALP and UPS, and processed for efficient elimination. In the UPS system, ubiquitinated substrates are accepted by ubiquitin receptors and subsequently de-ubiquitinated via Ubiquitin carboxyl-terminal hydrolase RPN11, which is key for substrate degradation. In the ALP system, in contrast to UPS, ubiquitin receptors form oligomers and facilitate substrate recognition via autophagosomal recruitment. Under AD or diseased conditions, a decrease in ALP and UPS mediates substrate degradation via downregulation of RPN11 and RPN11 mediated ubiquitination, thereby disrupting auto-phagosome recruitment for degradation. These changes might lead to the accumulation of pathological protein aggregates. RPN11, regulatory particle of non-ATPase subunit. [Reproduced from Bustamante, H, *et al.* with the permission of Front Cell Neurosci (194)].

2. HYPOTHESIS AND AIMS

Impairment in brain insulin signaling has been reported in AD in humans (73, 117, 123) and in AD mouse models (58, 143, 195). In the present thesis, we hypothesized that A β burden in AD may lead to impairment in cerebrovascular insulin response by modulating two potentially linked key receptors, LRP-1 and IR- β , involved in A β homeostasis and insulin signaling. To test our hypothesis, we employed the 3XTg-AD mouse model and for *in vitro* studies we used a BBB model composed of primary porcine brain capillary endothelial cells (pBCEC).

Aim 1: To understand the Impact of AD transgenes and HFD feeding on central and peripheral insulin signaling in 3XTg-AD mice.

We examined:

- the impact of AD and HFD on peripheral (liver) and central (brain) insulin signaling and metabolic profile.
- the impact of HFD feeding on memory and A β burden in 3XTg-AD mice.
- the impact of AD and HFD on peripheral (liver) and central (brain and cerebrovascular) key receptors (LRP-1 and IR- β) involved in insulin signaling.
- the impact of cerebral amyloid- β (A β) burden and HFD on central and cerebrovascular autophagy, and its correlation with changes in central and cerebrovascular expression of LRP-1 and IR- β receptors.

Aim 2: To understand the implications of A β burden on IR- β and LRP-1 homeostasis at the BBB, and on brain endothelial insulin signaling:

We investigated

- the impact of A β peptides on LRP-1 and IR- β protein expression and their interaction in pBCEC.
- the impact of A β peptides on autophagy and insulin signaling in pBCEC.
- potential pathways/mechanisms regulating A β mediated degradation of LRP-1 and IR- β in pBCEC.

3. MATERIALS AND METHODS

3.1 Chemicals and reagents used for cell culture

Purchased chemicals are listed in **Table 1**.

Table 1: Cell culture reagents and chemicals

Product	Company
75 cm ² cell culture flasks	Greiner Bio-One
6- and 12-well plates	Greiner Bio-One
Fisherbrand™ Superfrost™ Plus	Fischer Scientific
Transwell 12-well plates	Corning Life Sciences
Medium 199 (1x)	Life technologies
Minimum essential medium (MEM, 10x)	Life technologies
DMEM/ F-12 (Ham)	Life technologies
Dispase	Life technologies
Collagen G from bovine calf skin	M&B Stricker
Penicillin/streptomycin/gentamycin	PAA Laboratories
Trypsin- ethylenediaminetetraacetic acid (EDTA)	PAA Laboratories
Horse serum	PAA Laboratories
L-glutamine	PAA Laboratories
Collagenase/dispase	Roche
Hydrocortisone	Sigma- Aldrich
Percoll® pH 8.5- 9.5	Sigma- Aldrich
Dextran	VWR

3.1.1 Phosphate buffer saline (PBS)

Forty g NaCl, 1.5 g KCl, 0.1 g KH₂PO₄, 0.457 g Na₂HPO₄ (2 H₂O) and 10 g glucose were weighed and dissolved in five liters of double distilled water (d₂ H₂O), filter sterilized and autoclaved.

3.1.2 Collagenase/dispase solution

Hundred mg collagenase/dispase was dissolved in 10 ml plating medium A, sterile filtered through a 0.2 µm pore size nylon wool filter, 350 µl aliquots were made and stored at – 20 °C.

3.1.3 Dextran solution

Two hundred g dextran, 2.4 g NaHCO₃ and 109.1 ml MEM (10x) were dissolved in 1.2 L of d₂H₂O, stirred overnight at 4°C to dissolve, density was adjusted to 1.0612. The solution was autoclaved and stored in 500 ml glass bottles at 4 °C.

3.1.4 Percoll® biphasic solution

- **1.03 g/ml density solution:** to 40 ml 1 X PBS, 9 ml Percoll® pH 8.5- 9.5 and 1 ml DMEM (1x) were added and stored in 50 ml falcon tubes at 4 °C.
- **1.07 g/ml density solution:** To 20 ml 1 X PBS, 27 ml Percoll® pH 8.5- 9.5 and 3 ml DMEM (1x) were mixed and were stored as 50 ml aliquots at 4 °C.

3.1.5 Hydrocortisone solution (50 µg/ml)

One mg hydrocortisone was dissolved in 1 ml ethanol and 19 ml DMEM (1x) medium to attain 50 µg/ml stock solution, 500 µl aliquots were stored at – 20 °C until use.

3.1.6 Collagen G solution for coating flasks and cell culture plates

Mix 450 µl (for 60 µg/ml) or 900 µl (for 120 µg/ml) of Collagen G stock solution (4 mg/ml) with 30 ml PBS (1x) freshly for every coating experiment.

3.1.7 Preparation medium

To 500 ml medium M199 (1x) medium, 1% penicillin/ streptomycin, 1% gentamycin and 1 mM L-glutamine were added and stored at 4 °C.

3.1.8 Plating medium A

To 500 ml M199 (1x) medium, 10% horse serum, 1% penicillin/ streptomycin, 1% gentamycin and 1 mM L-glutamine were added and stored at 4 °C.

3.1.9 Plating medium B

Plating medium A without 1% gentamycin.

3.1.10 Serum free (SF) medium

To 500 ml Medium M199 (1x), 1% penicillin/ streptomycin and 1 mM L-glutamine were added and the resulting medium was stored at 4 °C.

3.1.11 DMEM/Ham's F12 medium

DMEM/Ham's F12 Medium was prepared by adding 1% penicillin/ streptomycin, 0.7 mM L-glutamine and 0.4% Hydrocortisone stock solution [50 µg/ml] to 500 ml of DMEM/Ham's F12 (1x) and stored at 4 °C.

3.1.12 Insulin stock solution (10 mM)

Dissolve 5.8 mg insulin in 100 µl of 0.01 N HCl and dilute (1:1000) the stock to obtain the working stock solution (10 µM). Stock solution is stable for up to 4 weeks at 4 °C.

3.1.13 Amyloid-β peptide stock solution

One mg recombinant human amyloid-beta peptide (Aβ₁₋₄₀ and Aβ₁₋₄₂) vials were purchased from Rpeptide, USA, as hexafluoroisopropanol (HFIP) dissolved films. Each 1 mg vial was dissolved in 1 ml of 1% NH₄OH, vortexed for 30 s until the film was invisible. Twenty µl aliquots of 1 µg/µl Aβ stocks were made, stored at -20°C and were used within 1 month.

3.1.14 MG132 stock solution (10 mM)

Five mg MG132 (Selleckchem) was dissolved in 1 ml dimethyl sulfoxide (DMSO) to attain 10 mM stock solution, 50 µl aliquots were stored at -20°C for further use.

3.1.15 Chloroquine stock solution (30 mM)

In 10 ml d₂H₂O, 154.8 mg chloroquine diphosphate salt was dissolved and sterile filtered through 20-micron nylon filter, 100 µl aliquots were stored at -20°C.

3.2 Chemicals and solutions used for RNA isolation, cDNA synthesis and quantitative Real time PCR (RT-qPCR)

All products and reagents used for RNA isolation and RT-qPCR are listed in **Table 2**. Pre-validated primers for RT-qPCR were purchased from Qiagen or self-designed and obtained from Life technologies (**Table 5**).

Table 2: Materials used for RNA isolation, cDNA synthesis and RT-qPCR

Product	Company
iQ SYBR® Green Supermix	Biorad
Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR Plates	Biorad
Microseal 'B' Adhesive Seals for PCR plates	Biorad
0.2 ml PCR Tubes	Biorad
C-1000™ Thermal Cycler	Biorad
Icycler iQ™, Real-time PCR Detection System	Biorad
Molecular Imager ChemiDoc XRS System	Biorad
Power Pac HC, Power Supply	Biorad
Biozym LE Agarose	Biozym
OneTouch Filter-tips, 10 µl	Biozym
Nuclease-free water	Carl Roth
HE 33 Mini Submarine Electrophoresis Unit	Hoefer® Inc.
High Capacity Reverse Transcriptase Kit	Life technologies
TriReagent	Molecular Research Center, Inc
2-Log DNA ladder	New England laboratories
Gel loading Dye, Blue (6x)	New England laboratories
NanoDrop® ND-1000 UV-Vis Spectrophotometer	Peqlab

3.2.1 RNA isolation from mouse tissue or endothelial cells

Cell culture plates were transferred on ice to a fume hood, 450 µl TRI reagent was added to each well, and contents were scraped and transferred into 1.5 ml Eppendorf tubes. For tissue samples (brain and liver) and microvessels (isolated from 4 pooled brain hemispheres), 1 ml/ ~40 mg (v/w) of TRI reagent was added

and samples were homogenized with an Ultra-Turrax homogenizer. Tubes were incubated at room temperature (RT) for 5 min to allow complete dissociation of nucleoprotein complexes. Fifty μ l 4-bromoanisole (BAN) phase separation reagent was added, tubes were vortexed for 10 s, and were incubated for 20 min at RT. After incubation, tubes were centrifuged at 12,000 g at 4 °C for 15 min. Following centrifugation, phase separation was achieved, as indicated by residual DNA, proteins, and polysaccharide precipitates pelleting in the organic phase at the bottom of tube, while RNA residues stay as soluble supernatant. Four hundred μ l of supernatant was carefully pipetted into a new tube without collecting the residuals (DNA) at the interface. Equal amount of isopropanol was added to each tube and RNA was precipitated for 10 min at RT. Next, tubes were centrifuged at 12,000 g for 10 min to pellet the RNA precipitate. Washing was performed by adding 500 μ l of 75% ethanol (v/v) to each tube and centrifuged at 8,000 g for 5 min, followed by a washing step with 75% ethanol (v/v) that was repeated once more to remove residual isopropanol contamination from the RNA pellet. The RNA pellet was dissolved in 60 μ l of RNase-free water and incubated for 10 min at 55 °C on a heat block while shaking to dissolve the pellet in water.

RNA concentrations were measured at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). Two μ l of RNA solution was used to determine the absorption. RNA concentration was calculated using the following equation:

$$c(\text{ngRNA}/\mu\text{l}) = \lambda_{260\text{nm}} \times 40$$

Only isolated RNA with 260/280 ratio of 1.7 to 2.1 and a 260/230 ratio of 1.6 to 2.3 were used for further cDNA preparation.

RNA integrity was determined by gel electrophoresis. Gels were prepared by boiling 1.1 g agarose, 11 ml 10x 3-(N-morpholino) propanesulfonic acid buffer (MOPS; 200 mM, 80 mM sodium-acetate, 10 mM EDTA, pH 7) and 88.8 ml nuclease free (NF) water in a microwave. After cooling down to approximately 50°C, 18.7 ml of formalin solution was added to the solution, casted into gel molds, and left to solidify at RT. Two μ g of RNA were mixed with 4 μ l of RNA loading buffer and were

ran at 70 V for 45 min. Band visualization was performed using GelRed® (Biotium) under UV light (ChemiDoc™ MP Imaging System). RNA was identified as non-degraded if two clear distinct bands were observed at 4.7 kb (28S rRNA band) and 1.9 kb (18S rRNA band).

3.2.2 cDNA preparation

Complementary DNA was prepared from RNA by reverse transcription. Two µg RNA in 10 µl (adjusted with NF d₂H₂O) were reversely transcribed by using High-Capacity DNA Reverse Transcription kit (Applied Biosystems, Foster City, CA).

Table 3: High-Capacity cDNA Reverse Transcription (master mix composition)

Component	Volume (ml)/reaction
10 x RT buffer	2
25 x dNTP mix (100 mM)	0.8
10 x RT random primers	2
Multiscribe reverse transcriptase	1
RNase inhibitor	0.7
NF H ₂ O	3.5
2 µg RNA/10 µl d ₂ H ₂ O	10
Total volume per reaction	20

Table 4: Reverse transcription thermal cycling conditions

Steps	S1	S2	S3	S4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 s	∞

3.2.3 RT-qPCR

For RT-qPCR analyses, cDNA (2000 ng/20 µl) was diluted 1:20 in NF d₂H₂O to achieve 5 ng/µl cDNA. Two µl of 5 ng/µl cDNA, 1 µl of forward and reverse primers (stock solution of 100 µM, diluted 1:10 with NF d₂H₂O), and 5 µl iQ™ SYBR® Green Supermix were pipetted into a Hard-Shell 96 well PCR plate.

Table 5: Primers used for RT-qPCR

Gene	Sequence (5'-3')	Amplicon size (bp)
ss HPRT1	F, AGGACCTCTCGAAGTGTG	247
	R, CAGATGGCCACAGGACTAGA	
ss LRP-1	F, AGGAGAACAGCAGGGGTGAA	97
	R, GGTGACGAGGTAAGTTCGGA	
ss IR- β	F, GGATTTTCGCTGGACTCTGC	153
	R, ACCCCTTTCCCTCTAGGTT	
mm CD31	F, AGGCTTGCATAGAGCTCCAG	278
	R, TTCTTGGTTTCCAGCTATGG	
mm CD13	F, CCCCAGGGGCTGCTGTTCTTT	1208
	R, ACCACCCGCTCCTTGTTGCTAATG	
mm GFAP	F, TCCTGGAACAGCAAAACAAG	224
	R, CAGCCTCAGGTTGGTTTCAT	
mm SYP	F, CATTGAGGCTGCACCAAGTG	60
	R, TGGTAGTGCCCCCTTTAACG	
mm MAP2	F, GCTGAGATCATCACACAGTC	211
	R, TCCTGCCAAGAGCTCATGCC	
mm IBA1	F, GGATTTGCAGGGAGGAAAAG	92
	R, TGGGATCATCGAGGAATTG	
mm AQP4	F, TGCCAGCTGTGATTCCAAACG	469
	R, GCCTTCAGTGCTGTCCTCTAG	
mm ZO1	F, GGAGAGGTGTTCCGTGTTGT	285
	R, GAGCGGACAAATCCTCTCTG	
mm PDGFR β	F, AGCTACATGGCCCCTTATGA	367
	R, GGATCCCAAAGACCAGACA	
mm HPRT1	F, GCCTAAGATGAGCGCAAGTTG	101
	R, TACTAGGCAGATGGCCACAGG	

sus scrofa (ss) and mus musculus (mm). [Reproduced from Gali CC., *et al*, (Supplementary Table 3) with the permission from Mol Cell Neurosci (1)]

Table 6: Real-time PCR mix

Reagents	Volume
iQ SYBR® Green Supermix	5 µl
cDNA	2 µl
Primer fw. (300 nM)	0.3 µl
Primer rev. (300 nM)	0.3 µl
Nuclease free water	0.4 µl
Total volume	10 µl

The 96-well plate was centrifuged for 1 min at 400 rpm to collect the mixture at bottom of the well and was loaded onto an Icyler iQ™, RT-qPCR Detection System (Biorad).

Table 7: RT-qPCR program conditions

Steps	Temperature	Time	Cycles
Denaturation	95°C	5 min	1
Amplification	95°C	10 s	40
	60°C	30 s	
Melting curve	95°C	10 s	1
	60°C	20 s	1
	95°C	continuous	
Cool Down	40°C	20 s	1

Hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used as house-keeping gene for relative gene expressions. The $2^{-\Delta\Delta CT}$ method was used to calculate relative gene expression of desired gene, later normalized to HPRT (196-198).

3.3 Silencing of LRP-1 and IR-β in pBCEC

Silencing of LRP-1 and IR-β in pBCEC was performed by using small interfering RNAs (siRNA) purchased from Microsynth and non-targeting control (NTC) siRNAs, purchased from Dharmacon Research (196, 197). DharmaFECT™ siRNA transfection reagent 1 (Dharmacon) was used, small interfering RNA used for silencing LRP-1 and IR-β were purchased from Microsynth and are listed in **Table 8**.

Table 8: siRNA sequences for LRP-1 and IR- β mRNA

siRNA	Sequence (5'-3')
LRP-1	GGAGGAUGACUGUGAACAU ACAACGCUGUCGCCUUGGA CCUGUACUGGUGUGACAAA
IR- β	GGGUCCGGAUAUAAAUUAA GAAAUGGAGUGCAUGUAAA ACACAGGCUUUGAUUAUAGA

[Reproduced from Gali CC., *et al*, (Material and methods, section 2.10) with the permission from Mol Cell Neurosci (1)]

Primary pBCEC were grown on 6-well plates or chamber slides, once they attained 80 - 90 % confluency, cells were transfected with LRP-1 or IR- β or NTC siRNA using transfection reagent (DharmaFECT), following the manufacturer's instructions. In brief, a pool of three siRNAs belonging to one gene were pooled together in NF water to attain a 5 μ M stock. Firstly, 50 nM siRNA was added to M199 1X medium without serum and antibiotics (tube A) and incubated for 5 min at RT. Secondly, 5 μ l DharmaFECT reagent was added to another tube containing M199 1X medium without antibiotics or serum (tube B) and incubated at RT for 5 min. After incubation, contents of tube A was added to tube B, mixed, and incubated for another 20 min at RT to form siRNA complexes with the transfection reagent. These complexes were added drop-wise to pBCEC incubated with M199 1X medium containing 10% horse serum. After 24 h, the medium was replaced with M199 1X medium containing 10% horse serum and 1% penicillin/streptomycin for another 24 h. At the end of (in total) 48 h incubation, pBCEC were harvested for mRNA and protein analysis. For insulin stimulation, after silencing, cells were serum starved for 12 h with SF medium. Cells were stimulated with 100 nM insulin by incubating for 30 min at 37 °C. After incubation, cells were placed on ice, washed with ice cold PBS, cells were lysed and proteins were extracted using protein lysis buffer (PLB). For immunofluorescence imaging, chamber slides were washed with PBS and were incubated for 10 min with 4% PFA, cells were washed with PBS 3 times, and slides were air dried and stored at -20 °C for further processing.

3.4 Protein isolation from cell and tissue lysates

Proteins from pBCEC were isolated by lysing the samples in PLB by sonicating the lysates in water bath with ice (3 min X 2 times), vortexing the tubes for 30 s and by centrifuging (Microcentrifuge, Qualitron DW- 41) the lysates at 12,000 g for 10 min at 4 °C. For isolating protein from tissue samples (liver and brain), 40 mg of tissue was lysed in 500 µl of PLB using ultra-turrax (3 x 30 s with 20 amplitude output on ice), and centrifuged at 12,000 g for 10 min at 4 °C. For both pBCEC and tissue lysates, supernatants containing proteins were collected in fresh tubes and pellets were discarded.

3.5 Determining protein concentration of cell or tissue lysates

To identify the protein concentration of tissue samples or cell lysates, Bicinchoninic acid assay (BCA) was performed. Bovine serum albumin (BSA) standards were prepared in PLB buffer from a BSA stock solution (2 mg/ml). A serial dilution of BSA standard was made to achieve concentrations from 2 mg/ ml to 0.125 mg/ml to attain a standard curve. Four µl of standard and protein samples was pipetted in replicates into a microplate well. BCA reagent A and B were mixed in a ratio 1:50, vortexed and 196 µl of the mixture is dispensed in the wells containing standards, samples and PLB blank. Plates were incubated for 30 min at 37 °C in a water bath. After incubation, absorbance was determined at 562 nm using a plate reader. Protein concentration of each sample was determined using the linear regression of the BSA standard curve.

3.6 Western blotting (WB)

Table 9: Buffers and reagents used for WB

Product	Company
XT sample buffer (4x)	Biorad
XT sample reducing agent (20x)	Biorad
Blotting-grade blocker, nonfat dry milk	Biorad
Clarity Western ECL Substrate	Biorad
IKA® Model MS1 minishaker	Carl Roth
Combi- Shaker KL 2	Carl Roth
Microcentrifuge, Qualitron DW- 41	Carl Roth

Concentrator Plus	Eppendorf
IKA® Model MS1 minishaker	Carl Roth
PhosSTOP™	Sigma Aldrich
ELMA Transsonic 460, ultrasonic unit	Carl Roth
Protran Premium 0.2 µm nitrocellulose (NC) membrane	Amersham
polyvinylidene difluoride (PVDF) membrane, Hybond-P	GE healthcare
Novex™ 16% Tricine Protein Gels	Thermo Scientific
NuPage® Novex 4-12% Bis- Tris Midi Gel	Life technologies
NuPage® MES SDS Running Buffer (20x)	Life technologies
NuPage® MOPS SDS Running Buffer (20x)	Life technologies
Pierce® BCA Protein Assay Kit	Thermo Scientific
BSA, 2 mg/ml	Thermo Scientific
ChemiDoc™ Imaging Systems	Biorad
Cell Proliferation Reagent WST-1	Roche
Sunrise photometer with Magellan software	Tecan
Centrifuge Sigma 3K15 with angle rotor 12154-H	Sigma
Protease Inhibitor Cocktail tablets	Sigma Aldrich
Magnetic stirrer with heating, MR 3001, Heidolph	VWR
Ratek RSM7, Rotary Suspension Mixer	VWR

3.6.1 Transfer buffer 10x (Towbin buffer)

Twenty-five mM Tris, 192 mM glycine, 20% (v/v methanol), 0.025% SDS were mixed to attain 10 x Towbin buffer (pH 8.3).

Table 10: composition of Towbin buffer

Compound	Amount
Tris base	58.15 g
Glycine	29.3 g
d ₂ H ₂ O	600 ml
0.025% SDS	20 ml
Adjust volume with d ₂ H ₂ O	Up to 1 L

One liter working solution was freshly made by mixing 100 ml, 10 x transfer buffer with 800 ml d₂H₂O and 200 ml methanol, stored at 4 °C for further use.

Table 11: Wash buffer (TBS-T) (pH 8.5)

Compound	Amount
Tris, (1 M; pH 7.5)	100 ml
Tween 20	2.5 ml
NaCl (2.5 M)	500 ml
d ₂ H ₂ O	Up to 5 L

3.6.2 Protein lysis buffer (PLB)

Fifty mM Tris, pH 7.5, 10 mM EDTA, 1% Triton-X-100, 1 tablet of protease inhibitor cocktail, 1 tablet of phosphatase inhibitor were dissolved in 5 ml of d₂H₂O, the final volume was adjusted to 10 ml with d₂H₂O and 1 ml aliquots were stored at -20 °C until use.

3.6.3 Blocking buffer

Blotting-grade blocker (non-fat dry milk) was dissolved in TBS-T to achieve 5% milk protein, also known as blocking buffer.

3.6.4 Sample preparation and SDS-polyacrylamide gel electrophoresis (PAGE)

Equal concentrations of protein lysates were mixed with XT loading dye and reducing agent to achieve a 1X final concentration, samples were heated on a heat block at 70 °C for 10 min to denature the proteins. Sample tubes were spun-down on a tabletop centrifuge to collect the mixture at the bottom of the Eppendorf tubes. Samples (15 or 20 µg of protein) were loaded onto 4-12% SDS-PAGE gels and electrophoresis was performed using MOPS/MES running buffer, for 150V for 60 min (MOPS) or 55 min (MES) to achieve proper separation of proteins.

3.6.5 Protein transfer from gel to membrane

Wet electrophoretic transfer of proteins to PVDF or NC membranes was done in a blotting chamber (Biorad); PVDF (but not NC) membranes were soaked in methanol for 60 s and then in transfer buffer before stacking the gel with the membrane (sandwich blot). Prior to building the sandwich in the cassette, filter papers and stacking sponges were soaked in transfer buffer for 10 min to remove any trapped air bubbles. Transfer sandwich was prepared in a chronological order starting with sponge, filter pads, membrane, gel, filter pads and finally sponge. The cassette was closed and any trapped air bubbles removed by using a roller during stacking the sandwich. In the transfer tank, the gel side of the cassette holder must face the cathode (-) while the membrane side must face the anode (+). Wet transfers was performed at 60 V for 1 h. For A β peptides, blotting to NC or PVDF 0.2 μ m membranes was done for 4 h at 15 V.

3.6.6 Ponceau S staining of proteins

Half a g of Ponceau S was dissolved in 25 ml acetic acid and the volume made up with d₂H₂O to 1 L. To stain the proteins immediately after the transfer, blots were incubated with 50 ml of fresh Ponceau S solution for 30 min at RT with gentle shaking. After 30 min incubation, blots were washed with tap water 3 times until the background was clear. Ponceau S images were taken using a ChemiDoc™ imaging system. Immediately, blots were washed with tap water 3 X 5 min with shaking at RT to destain Ponceau S. After destaining, blots were processed for blocking. For A β blots, NC membranes were heated in a microwave for 3 min to expose the epitopes.

3.6.7 Membrane blocking

Membranes were blocked using blocking buffer (pH 8.5) (section 1.6.3) for 1 h with constant gentle shaking at RT.

3.6.8 Primary antibody incubation

Primary antibodies were prepared in 5% nonfat dry milk protein or BSA (for phosphorylated proteins) dissolved in TBS-T (pH 8.5) according to the manufacturer

instructions. Blots were incubated overnight at 4 °C in a cold room with constant shaking.

3.6.9 Washing the blots

Washing was done using washing buffer (1x) (**Table 11**), 3 X 5 min at RT with constant shaking after every primary and secondary antibody incubations.

3.6.10 Secondary antibody incubation

Rabbit, mouse, and goat HRP labeled secondary antibodies were used. Antibody incubations were performed in 5% nonfat dry milk protein in TBS-T (pH 8.5) for 90 min at RT with constant shaking.

3.6.11 Signal detection on Western blots and semi-quantitative analysis

Signal detection was performed using Clarity Western ECL substrate. In brief, 13 X 7 cm blots were incubated with 2 ml ECL substrate mixture for 5 min at RT. Chemiluminescence was detected using a ChemiDoc™ imaging system. Signal quantification was performed by densitometry using ImageLab™ software, band intensities were normalized to corresponding band intensities of the housekeeping proteins GAPDH or β -actin.

Table 12: List of antibodies used for western blotting

Antibody	Clone	Host	Source	Dilution
anti-LRP-1 antibody (ab192308)	polyclonal	rabbit	Abcam	1:10000
phospho-AKT (Ser473)	polyclonal	rabbit	CST	1:2000
phospho- Insulin Receptor β (Tyr1150/1151)	monoclonal	rabbit	CST	1:3000
phospho-S6 ribosomal protein (Ser235/236) (2F9)	monoclonal	rabbit	CST	1:5000
phospho-IRS-1 (Ser1101)	polyclonal	rabbit	CST	1:1000
Total AKT	polyclonal	rabbit	CST	1:2000
IRS-1	monoclonal	rabbit	CST	1:1000

phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	polyclonal	rabbit	CST	1:2000
p44/42 MAPK (Erk1/2)	polyclonal	rabbit	CST	1:2000
Insulin Receptor- β (4B8)	monoclonal	rabbit	CST	1:1000
β -Actin	polyclonal	rabbit	Sigma-Aldrich	1:5000
β -Actin	polyclonal	rabbit	CST	1:5000
GAPDH	polyclonal	rabbit	CST	1:2000
anti-Rabbit LC3B	polyclonal	rabbit	CST	1:2000
LC3B (D11)	monoclonal	rabbit	CST	1:2000
S5a/PSMD4	monoclonal	rabbit	CST	1:1000
HSP27	monoclonal	rabbit	CST	1:1000
ATG3	polyclonal	rabbit	CST	1:2000
ATG5	monoclonal	rabbit	CST	1:2000
anti-mouse CD107a (LAMP-1)	monoclonal	rat	BioLegend	1:1000
anti- β -Amyloid (1-16) 6E10	polyclonal	mouse	BioLegend	1:2000
anti-Rabbit IgG-HRP	-	goat	CST	1:5000
anti-Mouse IgG-HRP	-	mouse	CST	1: 10000

CST: Cell Signaling Technologies. [Reproduced from Gali CC., *et al*, (Supplementary Table 1) with the permission from Mol Cell Neurosci (1)]

3.7 Animals & housing

All mice used in this study were bred in the animal facility of University of Graz. Animal breeding and experiments were approved by the Austrian Federal Ministry of Science and Research, Vienna, Austria (approval number 66.010/130-WF/V/3b/2015). Mice were maintained under standard housing conditions with controlled humidity, temperature, free access to water, and in a regular light-dark cycle (12 h light, 12 h dark). Male and female 3XTg-AD (PS1^{M146V}, APP^{Swe}, and tau^{P301L}) and NTg (C57/BL6) mice were used in this thesis (1, 199).

3.8 Genotyping

Genomic DNA was isolated from tail tips of 3XTg-AD mice, carrying mutations for the human swedish mutation of amyloid precursor protein (APP_{Swe}), presenilin 1 (PS1_{M146V}) and tau_{P301L} (200), using Phire Animal Tissue Direct PCR Kit (F140WH Thermo Scientific), following the manufacturer's instructions. PCR runs were performed using Phire Hot Start II DNA Polymerase (F122L) to amplify human APP_{Swe} and PS1_{M146V} transgenes using primer templates provided by the Jackson Laboratory. PCR products were digested with Bste II (NEB R0162) in NEB Buffer 3 (B7203) at 60 °C for 3 h, PCR products were separated on 1.5% agarose gels along with wild type (WT) and water (H₂O) controls. Band visualization was performed using peqGREEN (Peqlab Biotech) under UV light using ChemiDoc™ MP Imaging System (1).

Table 13: Genotyping for APP

Reagent	Amount (µl)
H ₂ O	10.8
dNTP's 10mM	0.4
5x PCR buffer	4
APP forward primer	0.6
APP reverse primer	0.6
internal forward primer	0.6
internal reverse primer	0.6
Hot start polymerase II	0.4
DNA	2

Table 14: Genotyping for PS1

Reagent	Amount (µl)
H ₂ O	10
dNTP's 10mM	0.4
5x PCR buffer	4
PS1 forward primer	0.6
PS1 reverse primer	0.6
Hot start polymerase II	0.4

DNA	4
-----	---

Table 15: Bste II digest preparation

Reagent	Amount (µl)
H ₂ O	7
10x NEB3 buffer	2
Bste II	1
PCR product (PS1)	10
Digest: 3 h at 60°C	

Table 16: Cycle conditions used for DNA amplification

	APP		PS1	
Initial denaturation	98 °C (3 min)			
Denaturation	98 °C (30 sec)		94 °C (3 min)	
Annealing	52 °C (1 min)	34 X	94 °C (30 sec)	34 X
Elongation	72 °C (1 min)		64 °C (1 min)	
Final elongation	72°C (3 min)		72 °C (1 min)	
Cool down	4°C – ∞			

3.9 Feeding timeline

Non transgenic (NTg) and 3XTg-AD mice were fed either HFD containing 60% kcal from fat (D12492, ssniiff® diets, Germany; Table 17) or standard chow diet containing 12% kcal from fat, for 23 weeks starting from 12 weeks after weaning (8-10 mice per group/sex). Body weights were recorded weekly (1).

Table 17: Composition of high fat diet (Ssniff® diets)

Crude Nutrients	%
Crude protein	24.4
Crude fat	34.6
Crude fiber	6.0
Crude ash	5.3
Starch	0.1
Sugar	9.4

N free extracts	26.3
Minerals	%
Calcium	0.92
Phosphorus	0.64
Calcium/phosphorus	1.44
Sodium	0.20
Magnesium	0.23
Potassium	0.97
Fatty acids	%
C 12:0	0.07
C 14:0	0.44
C 16:0	7.93
C 18:0	4.37
C 20:0	0.11
C 16:1	0.94
C 18:1	13.97
C 18:2	4.64
C 18:3	0.49
Amino acids	%
Lysine	2.02
Methionine	0.86
Cysteine	0.45
Met+Cys	1.31
Threonine	1.07
Tryptophan	0.33
Arginine	0.95
Histidine	0.74
Valine	1.70
Isoleucine	1.38
Leucine	2.42
Phenylalanine	1.27
Phe+Tyr	2.56
Glycine	0.52

Glutamic Acid	5.50
Aspartic Acid	1.82
Proline	2.80
Serine	1.46
Alanine	0.81
Vitamins	Per Kg
Vitamin A	15,000 IU
Vitamin D3	1,500 IU
Vitamin E	150 mg
Vitamin K	20 mg
Thiamine (B1)	25 mg
Riboflavin (B2)	16 mg
Pyridoxine (B6)	16 mg
Cobalamin (B12)	30 µg
Nicotinic acid	47 mg
Pantothenic acid	55 mg
Folic acid	16 mg
Biotin	300 µg
Choline	1,140 mg
Trace elements	Per Kg
Iron	168 mg
Manganese	95 mg
Zinc	65 mg
Copper	13 mg
Iodine	1.2 mg
Selenium	0.2 mg

3.10 Oral glucose tolerance test

For OGTT, blood was drawn through the tail vein of mice fasted for 6 h before (0 min) and after 15, 30, 60, 120 min of oral gavage of 2 g of d-glucose/kg body weight. Blood glucose levels were measured immediately using Glucometer Accu-check-active strips (Roche Diagnostics) (1).

3.11 Barnes Maze study

The Barnes Maze (BM) test was performed as previously described (201). Briefly, mice were positioned on an elevated round wooden board with a diameter 90 cm and with 20 equally spaced holes (diameter: 5 cm) towards the edge. As depicted in (**Fig. 15**), mice were habituated to this maze (day 1) and then trained to find a target hole connected to a hidden, black escape box (11 x 5 x 5 cm) beneath the maze for 3 days (2 trials/day). The latency to find the target hole, as well as the number of primary errors were used to assess learning capabilities. The latency to approach the first hole was used to identify any influence of locomotor or anxiety-like behavior. Forty-eight h after this acquisition phase, the escape box was removed, the time spent in each target quadrant was used as a memory readout. The total distance traveled was measured to evaluate locomotion and exploration. The behavior of the animals during the 2 min learning and probe trials was videotaped and tracked with VideoMot 2 software (TSE Systems, Bad Homburg, Germany).

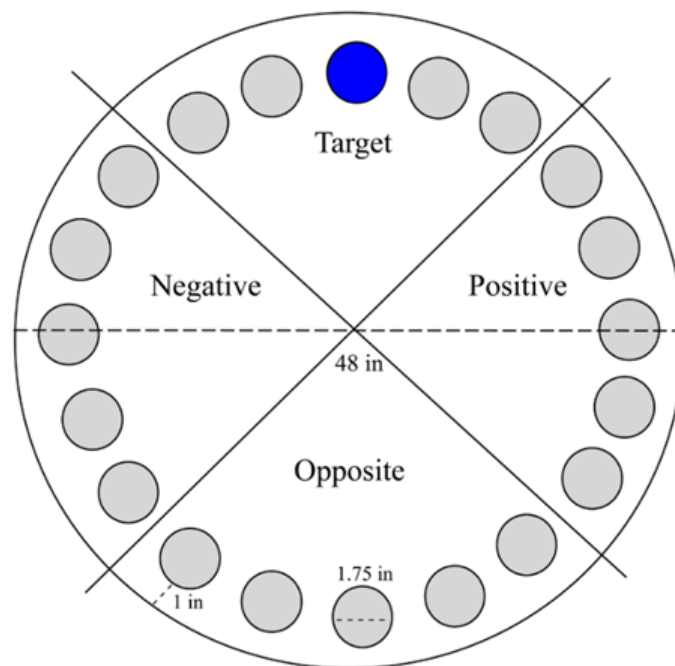


Figure 15: Diagrammatic representation of Barnes Maze with quadrants.

The Barnes maze is an elevated circular platform, which is 48 inches in diameter, with 20 equally spaced symmetric holes around the periphery. The holes are positioned 1 inch inward from the edge and have a 1.75-inch diameter. The maze is divided into 4 quadrants, labeled as target, positive, opposite, and negative. An escape hole is located at the center

of the target quadrant (in blue). [Reproduced from Attar A., et al. with the permission of PLoS One (201)]-

3.12 Harvesting primary organs and blood

On the final day of feeding, animal weights were recorded. Six hour fasted mice were euthanized under CO₂ flow. Via cardiac puncture ~ 500 µl blood was collected in tubes with 10% 0.5M EDTA for further processing. Immediately, vital organs such as brain, liver, and abdominal adipose tissue were extracted and stored at -80 °C. Four brain halves from each mouse cohort were pooled together and were processed for mBCEC isolation.

3.13 Plasma isolation

Plasma was isolated from fresh blood collected in 10% 0.5M EDTA Eppendorf tubes. Tubes were centrifuged at 2000 g for 15 min at RT. Supernatant containing plasma was carefully aspirated into fresh Eppendorf tubes, 50 µl aliquots were made and stored at -80 °C for later use.

3.14 Quantitative analysis of cholesterol, triglycerides and non-esterified fatty acids in plasma samples

Plasma triglycerides, cholesterol, and non-esterified fatty acid concentrations were measured using enzymatic test kits (Triglycerides (TG); Cholesterol FS and non-esterified fatty acids (NEFA FS); DiaSys, Holzheim, Germany) according to the manufacturers' instructions.

Table 18: Assay conditions for triglycerides and/or cholesterol

	Blank	Sample or standard
Sample or standard	-	2 µl
d ₂ H ₂ O	2 µl	-
Reagent	100 µl	100 µl
Mix, incubate 20 min at 25°C, read absorbance against the blank within 60 min.		

$$\Delta A = (A_2 - A_1) \text{ sample/standard}$$

Wavelength: 500 nm, Hg 546 nm

Optical path: 1 cm

Temperature: 20 – 25°C/37°C

Measurement: against reagent blank

Calculation (with standard or calibrator):

$$\text{Triglycerides (mg/dl)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{concentration of standard (mg/dl)}$$

Conversion factor for triglycerides and cholesterol:

Triglycerides [mg/dL] x 0.01126 = triglycerides [mmol/L]

Cholesterol [mg/dL] x 0.02586 = cholesterol [mmol/L]

Table 19: Assay conditions for NEFA

	Blank	Sample or standard
Sample or standard	-	2 µl
d₂H₂O	2 µl	-
Reagent 1	100 µl	100 µl
Mix and incubate for 5 min. Read absorbance A1, then add reagent 2		
Reagent 2	25 µl	25 µl

$\Delta A = (A_2 - A_1)$ sample/standard

Wavelength: 546 nm/600 nm (bichromatic)

Optical path: 1 cm

Temperature: 37°C

Measurement: against reagent blank

Calculation:

$$\text{NEFA (mg/dl)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{concentration of standard (mg/dl)}$$

Conversion factor:

NEFA [mg/dL] x 0.0354 = NEFA [mmol/L]

3.15 Quantification of plasma insulin levels

Plasma insulin levels were determined using Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, USA), following the manufacturer's guidelines.

Table 20: Insulin ELISA method

Steps	Procedure
1	95 µl diluent was mixed with 5 µl sample in a 96 well ELISA plate
2	ELISA plates were incubated for 2 hours at 4 °C
3	Plates were washed three times with washing buffer
4	100 µl conjugate solution was added to each well
5	Plates were incubated for 30 min at RT
6	Plates were washed three times with washing buffer
7	100 µl substrate solution was added to each well
8	Plates were Incubated for 40 min at RT
9	100 µl of stop solution was added to each well
10	OD measurements were recorded at 450/630 nm

The insulin concentration (ng/ml) in each sample was determined using a standard curve method.

3.16 Extraction of soluble and insoluble A β from mouse brain

[Materials and equipment required for A β extraction were listed from (202) with the permission of Bio Protoc]

Table 21: Materials and equipment required for A β extraction from mouse brain

Materials	Company
1.5 ml open-top Eppendorf tubes	Thermo Scientific
Diethylamine (DEA) ($\geq 99.5\%$)	Sigma-Aldrich
95% formic acid (FA)	Carl Roth
100 mM NaCl	Sigma-Aldrich
Tris base	Thermo Scientific
0.5 M sodium phosphate dibasic (Na ₂ HPO ₄)	Carl Roth
0.05% sodium azide (NaN ₃)	Thermo Scientific
250 mM sucrose	Thermo Scientific
0.5 mM EDTA, disodium salt dihydrate	Thermo Scientific
0.5 mM Ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA)	Thermo Scientific

Protease Inhibitor Cocktail	Sigma-Aldrich
Beckman Coulter Optima L-90K Ultracentrifuge with a SW50.1 rotor	Beckman
Ultra-turrax, T10	IKA
Ultrasonic sonicator, ELMA Transsonic 460	Carl Roth

3.16.1 Reagents required

[Reagents required for A β extraction were cited from (202) with the permission of Bio Protoc]

0.4% Diethylamine (DEA) solution

Table 22: Materials and equipment required for preparing DEA solution

Ingredient	Amount
DEA	200 μ l
5 M NaCl	1 ml
d ₂ H ₂ O	Up to 50 ml

Stored at RT and used within 3 months

Table 23: Formic acid neutralization buffer (FANB) composition

Ingredient	Amount
1 M Tris base	12.1 g
0.5 M Na ₂ HPO ₄	7.1 g
0.05% NaN ₃	0.05 g
d ₂ H ₂ O	Up to 100 ml

Stored at RT and used within 3 months

Table 24: Tissue homogenization buffer (THB) composition

Ingredient	Amount
2 mM Tris (pH 7.4)	0.024 g
250 mM sucrose	8.5 g
0.5 mM EDTA	0.014 g
0.5 mM EGTA	0.019 g
RNase-free H ₂ O	Up to 100 ml

Protease inhibitor cocktail (100X) 100 μ l added freshly to 10 ml THB.
--

Stored at 4 °C and used within 3 months

3.16.2 Diethylamine neutralizing buffer (DEANB) or 0.5 M Tris-HCl (pH 6.8)

Table 25: DEANB composition

Ingredient	Amount
Tris-hydrochloride	6.05 g
RNase-free H ₂ O	Up to 100 ml
pH adjusted to 6.8	

Stored at RT and used within 4 weeks

3.16.3 Procedure for DEA and FA extraction of A β products

Each mouse brain hemisphere was mechanically homogenized in 850 μ l cold THB buffer using an Ultra-turrax for 3 X 30 s until it became a homogenous mixture. Resulting mixture was separated into three 250 μ l aliquots for DEA/FA processing. To each 250 μ l homogenate, 250 μ l 0.4% DEA was added and vortexed for 30 s. This mixture was transferred into Eppendorf tubes (ultracentrifuge grade) and tubes were centrifuged at 135,000 g for 1 h at 4 °C. After centrifugation step, from each tube withdraw 425 μ l supernatant into a fresh tube and neutralize it with 42.5 μ l of DEANB. Aliquot and store them at -80 °C for further use. To the pellet, add 125 μ l of cold FA solution and sonicate each sample for 3 X 30 sec with 20 amplitude output on ice, and centrifuged at 109,000 g for 1 h at 4 °C. Carefully, 105 μ l of supernatant was extracted into a new tube and neutralized with 1.895 ml of FANB. The resultant mixture was vortexed and aliquoted into Eppendorf tubes for further processing (202). BCA assays for DEA/FA extracts were performed to determine protein contents of samples (1).

3.17 A β ₁₋₄₂ and A β ₁₋₄₀ ELISA

Amyloid- β peptide levels in DEA and FA extracts were quantified using enzyme-linked immunosorbent assay (ELISA) kits from ThermoFisher Scientific (catalogue # KHB3441 and KHB3481), according to the manufacturer's indications.

Table 26: Contents of A β ₁₋₄₂ and A β ₁₋₄₀ ELISA kits

Contents	Amount
Human A β ₁₋₄₂ / A β ₁₋₄₀ Standards, (lyophilized synthetic peptide; with 0.1% sodium azide)	1 vial
Standard diluent buffer; with 0.1% sodium azide	60 ml
Antibody coated plate, 96-well plate	1 plate
Human A β ₁₋₄₂ / A β ₁₋₄₀ detection antibody; with 0.1% sodium azide	6 ml
Anti-Rabbit IgG HRP (100X)	0.125 ml
HRP diluent; contains 3.3 mM thymol	25 ml
Wash buffer concentrate (25X)	50 ml
Stabilized chromogen, tetramethylbenzidine (TMB)	25 ml
Stop solution	25 ml
Plate covers	2

3.17.1 Buffers required for ELISA assay

3.17.1.1 Standard reconstitution buffer

Sodium bicarbonate (2.31 g) was dissolved in 500 ml d₂H₂O; pH was adjusted to 9.0 using 2 N sodium hydroxide (NaOH) solution and was sterile filtered using a 0.2 μ M filter unit.

3.17.1.2 Washing buffer

Sixteen ml washing buffer concentrate (25X; proprietary composition) was diluted with 384 ml of d₂H₂O and labeled as 1X wash buffer.

3.17.1.3 1X Anti-Rabbit IgG HRP solution

For each 8-well strip used in the assay, 10 μ l anti-rabbit IgG-HRP (100X) was mixed with 1 ml HRP Diluent.

3.17.2 ELISA assay and measurement

All the reagents were brought to RT before starting the experiment. Fifty μ l of standards, controls, or samples were pipetted into the appropriate wells, chromogen blank wells were kept empty. Fifty μ l of Human A β ₁₋₄₀/A β ₁₋₄₂ detection antibody was

added to all wells except the chromogen blanks. Plates were tapped on the side, covered and were incubated at RT for 3 h. After incubation, plates were washed with 1X washing buffer three times. 100 μ l anti-rabbit IgG HRP was added to each well except chromogen blanks. Plates were covered and incubated for 30 min at RT. Plates were washed 4X with 1X washing buffer. Hundred μ l stabilized chromogen was added to each well and incubated for 30 min at RT in dark. After incubation, 100 μ l stop solution was added to each well, a change in color from blue to yellow is observed in standards and samples containing $A\beta_{1-40}/A\beta_{1-42}$. Absorbance was read at 450 nm. A 4 parameter algorithm was used to generate the standard curve and concentrations of unknown samples and controls were calculated from the standard curve.

3.18 Isolation of murine brain microvasculature

Brain microvasculature was isolated freshly from 3-4 pooled brain hemispheres, which were devoid of large capillaries and meninges (197, 203). In brief, mouse brain hemispheres were rolled on a paper towel to get rid of major blood vessels and meninges, and were cut into tiny pieces using a scalpel blade in 1 ml of MCDB131 medium containing 2% FBS, 1% L-glutamine and 1% penicillin/streptomycin. Contents were transferred to a glass douncer (5 ml) with a glass piston with high clearance. Tissue was homogenized by moving the piston five times until the solution was homogenous. Contents from the douncer were transferred to a sterile glass beaker with a magnetic stirring bar, final volume was adjusted to 5 ml with MCDB131 medium containing 0.02 g dispase. Enzymatic digestion was allowed to occur in a water bath at 37 °C for 1 h with constant stirring at low speed. Reaction was stopped by adding equal amount of cold dextran (1.0612 g/L) solution. The resultant mixture was centrifuged at 6800 g for 10 min at 4 °C. The supernatant with myelin was discarded and the pellet containing microvasculature were suspended in MCDB131 medium with 2% FBS, 1% L-glutamine and 1% penicillin/streptomycin. The suspension was strained through a nylon mesh with 180 μ m pore size to separate microvasculature from contaminants. Separated microvessels were centrifuged at 900 rpm for 5 min to pellet, resuspended in MCDB131 medium and were layered onto a percoll gradient (percoll reagent B (1.03 g/ml) layered over percoll reagent A (1.07 g/ml)) and centrifuged at 4000 g for 10 min at 4 °C in a swinging bucket centrifuge. Microvessels were collected from the percoll interface and were washed in

cold 1X PBS. Microvessel pellets were either stored at -80 °C for further processing or were smeared onto glass slides, for subsequent immunofluorescent (IF) staining (1).

3.19 Isolation of porcine brain capillary endothelial cells (pBCEC)

pBCEC were isolated from 3-4 fresh pig brain hemispheres obtained from the local slaughter house (196, 203). All the glassware and stainless steel equipment was autoclaved and UV irradiated prior to initiation of the isolation. Firstly, meninges and larger surface cerebral vessels were removed using a pair of forceps. Clean brain hemispheres were then cut into 1 cm pieces using a scalpel, and minced into very fine bits using a roller blade (cutter); excess myelin was removed using forceps. Minced brain was transferred into a glass jar with a magnetic stirrer bar. Dispase digestion was performed by dissolving ~ 70 mg dispase in preparation medium, and adding the dispase solution to the tissue to obtain a final volume to 150 ml. The glass jar with the brain tissue and medium with dispase was closed tightly with a lid and was incubated at 37 °C in a water bath for 1 h with continues stirring. After 1 h incubation, dispase reaction was neutralized by adding equal amount (150 ml) of density adjusted cold dextran (1.0612 g/ml) solution. The mixture was equally dispensed into ultra-centrifugation tubes and the suspension was centrifuged at 6800 g for 10 min at 4 °C. The supernatant with dense myelin was discarded and the endothelial cell pellets were suspended in medium A. Microcapillaries were separated from the larger capillaries by filtering the suspension through a nylon mesh with 180 µm pore size. The obtained microcapillaries were carefully enzymatically disrupted by adding 350 µl collagenase/dispase solution and incubating for 1-10 min in the 37°C water bath under slowest possible stirring to avoid generation of shear stress that leads to separation into single cells. Degree of digestion was checked under the microscope and stopped immediately by adding medium A when predominantly capillary fragments of several cells became apparent. The suspension was centrifuged at 900 rpm for 5 min at RT to pellet the micro capillaries, supernatant was discarded and the pellet was resuspended in 5 ml medium A. Capillary suspension was carefully overlaid onto a percoll bi-phase gradient, which was composed of 15 ml of 1.07 g/ml percoll solution on bottom and 20 ml of 1.03 g/ml percoll solution on top, and centrifuged at 1300 g for 10 min at RT in a swinging bucket rotor to separate the microcapillaries from contaminating cells and residual myelin. Using a

pasteur pipette, capillaries at the interface of the percoll bi-phase gradient were collected and transferred into a 50 ml Falcon tube with 35 ml Medium A. Cells were pelleted at 900 RPM for 5 min at RT in a swinging bucket centrifuge to get rid of excess percoll bi-phase and were resuspended in Medium A. Cells were plated on to collagen coated 75 cm² cell culture flasks containing medium A and were incubated at 37 °C in humidified air containing 5% CO₂. Twenty-four hours after incubation, adherent endothelial cells were washed twice with 1 X PBS and changed to culture medium B until they grew 70-90% confluent. After reaching ~ 90% confluency, cells in the flasks were carefully trypsinized and were seeded onto collagen coated 6-well plates and 12-well plates (coated with 60 µg/ ml collagen) or Superfrost™ Plus chamber slides (coated with 120 µg/ml collagen for 1 h at 37 °C in humidified air containing 5% CO₂) for subsequent experiments (1).

3.20 Congo red staining to detect amyloid plaques and fibril deposits in mouse brain sections

Congo red stainings were performed on 3XTg-AD mouse brain hippocampal cryosections to identify signs of CAA in the cerebrovasculature (204).

3.20.1 Reagents required for Congo red staining

3.20.1.1 Modified Weigert's iron hematoxylin

Solution A: hematoxylin (2 g) was dissolved in 100 ml of 80% ethanol.

Solution B: 4 ml 62% ferric chloride was dissolved in 95 ml d₂H₂O, and 1 ml HCl was added to the final solution.

Equal proportions of solutions A and B were mixed together.

3.20.1.2 0.5% acid alcohol

To attain 0.5% acid alcohol solution, 0.5 ml HCl was added to 99.5 ml of 70% alcohol.

3.20.1.3 2.5% sodium chloride (NaCl-) alcohol

To make 2.5% NaCl- alcohol solution, 2.5 g of NaCl was dissolved in 50 ml d₂H₂O, and then 50 ml absolute alcohol was added.

3.20.1.4 1% sodium hydroxide solution

One gram NaOH was dissolved in 100 ml d₂H₂O.

3.20.1.5 Alkaline Congo red

Congo red solution was prepared by dissolving 0.2 g of Congo red in 100 ml NaCl-alcohol solution, and then 1 ml 1% NaOH was added. This solution was filtered to remove undissolved precipitates.

3.20.1.6 Congo red staining procedure and microscopy

Glass slides with 18 µm brain cryosections were brought to RT and were fixed with 4% PFA for 10 min. Slides were then rinsed 3 X with d₂H₂O and were placed in modified Weigert's iron hematoxylin for 2 s. After hematoxylin incubation, slides were incubated with NaCl-alcohol solution for 5 s. Slides were then rinsed 2 X with d₂H₂O, then submerged in a container with 95 % ethanol for 5 s. Immediately after ethanol treatment, slides were transferred to a beaker filled with congo red solution and were incubated for 20 min. Sections were cleared by washing the slides in 3 changes of xylene, and sections were mounted with synthetic resin using a coverslip. Bright field images of Congo stain were acquired using a BX53 Upright Microscope (Olympus) equipped with XM10 Video camera (Olympus). Bi-refrangent images of Congo red stain were obtained using a polarizing microscope (Axio Scope.A1; Carl Zeiss MicroImaging GmbH) (1).

3.21 Immunofluorescent staining**3.21.1 IF staining of snap frozen mouse brain sections**

Studies were carried out on 18 µm cryosections of mouse brain samples. Prior to tissue fixation, sections were taken out from -20 °C and warmed up to room temperature for 20 min. Tissue sections were then fixed with 4% PFA for 10 min, rinsed in distilled water prior to IF staining. All incubations were performed at RT. Sections were washed with TBS-T(pH7.4), blocked with donkey serum for 30 min before incubating with primary antibody for 1 h at room temperature. The antibody concentration for mouse anti-β-Amyloid (6E10) was 5.0 µg/ml. All incubation steps were performed in a dark, moist chamber at RT. After 5 min of TBS-T wash, secondary antibody was applied: donkey anti-mouse Cy-3 (red) 1.88 µg/ml (Jackson

ImmunoResearch Lab, Inc. PA, USA) for 30 min. After another TBS-T wash, DAPI was added to the slides for 20 min as a nuclei counter stain. Sections were rinsed with TBS-T before mounting with Vectashield mounting medium (Vector Lab, Inc., Burlingame, CA, USA) (1).

3.21.2 Double-label IF staining of heat fixed brain microvessels

Slides with heat fixed microvessels were taken out from -20°C and were brought to RT for 20 min. Slides were then fixed with 4% PFA for 10 min, rinsed 2 X with d₂H₂O prior to IF staining. All incubations were performed at RT. Slides were washed with TBS-T pH 7.4, blocked with 100 µl donkey serum for 30 min preceding to primary antibody incubation for 1 h. Slides were incubated with rabbit anti-LRP-1 antibody (ab192308, 0.8 µg/ml) and mouse anti-human IR-β (CT-3, 1.00 µg/ml); rabbit anti-human ZO-1 (1.0 µg/ml) and mouse anti-human CD31 (1.4 µg/ml); mouse anti-human CD31 (1.4 µg/ml) and rabbit anti-LRP-1 antibody (ab192308, 0.8 µg/ml); mouse anti-human CD31 (1.4 µg/ml) and rabbit insulin receptor-β (4B8, 1.1 µg/ml); rabbit anti-human ZO-1 (1.0 µg/ml) and mouse anti-human insulin receptor-β (CT-3, 1.0 µg/ml); rabbit anti-human ZO-1 (1.0 µg/ml) and mouse anti-human LRP-1 (1.0 µg/ml). Negative controls were incubated with the mouse and rabbit IgG fractions as isotope controls. All incubations were done in a dark, moist room at RT. After 1 X 5 min TBS-T wash, secondary antibodies were applied: donkey anti-rabbit A594 (red) 2.0 µg/ml and donkey anti-mouse Dylight 488 (green) 1.67 µg/ml for 30 min. After 1 X TBS-T wash, slides were incubated with DAPI and incubated for 20 min as a nuclear counter stain. Sections were rinsed again with 1 X TBS-T and were mounted with Vectashield medium (1).

3.21.3 IF staining of pBCEC

IF stainings were carried out on pBCEC chamber slides. Slides were taken out from -20 °C and were brought to room temperature. Slides were fixed with 4% PFA for 10 min, slides were rinsed with d₂H₂O, all incubations were done at RT. Slides were washed with TBS-T, and were blocked with donkey serum for 30 min before primary antibody incubation. Slides were incubated for 1 h at RT with primary antibodies: (double IF stainings: rabbit anti-LRP-1 antibody (ab192308, 0.8 µg/ml) and mouse anti-human insulin Receptor-β (CT-3, 1.0 µg/ml); anti-mouse CD107a

(LAMP-1, 5.0 µg/ml) and rabbit anti-LRP-1 antibody (ab192308, 0.8 µg/ml); anti-mouse CD107a (LAMP-1, 5.0 µg/ml) and rabbit anti-IR-β (4B8, 1.0 µg/ml), and IF stainings: LC3B (D11, 3.6 µg/ml); rabbit anti-LRP-1 antibody (ab192308, 0.8 µg/ml); anti-mouse CD107a (LAMP-1, 5.0 µg/ml) and rabbit anti-IR-β (4B8, 1.0 µg/ml)). All incubations were performed in a dark, moist chamber at RT. Five minutes after TBS-T wash, secondary antibodies were applied: donkey anti-mouse Cy-3 (red) (1.88 µg/ml), Alexa Fluor 594 donkey anti-rabbit IgG (2.0 µg/ml) and Alexa Fluor 488 donkey anti-mouse IgG (1.7 µg/ml) for 30 min. This was followed by another TBS-T wash, and slides were incubated with DAPI as nuclei counter stain. Porcine BCEC-slides were rinsed once with TBS-T and were mounted with Vectashield (1).

3.21.4 Detection of IF stainings

To acquire and analyze digital IF images of mouse brain sections and pBCEC cells, a Leica DM4000 B microscope (Leica Cambridge Ltd.) equipped with Leica DFC 320 Video camera (Leica Cambridge Ltd.) was used.

Table 27: List of antibodies used for IF staining

Antibody	Clone	Host	Source	Concentration
rabbit anti-LRP-1 antibody (ab192308)	polyclonal	rabbit	Abcam	0.8 µg/ml
rabbit insulin receptor-β (4B8)	monoclonal	rabbit	CST	1.0 µg/ml
mouse anti-human β-Amyloid, 1-16 (6E10)	monoclonal	mouse	BioLegend	5.0 µg/ml
mouse anti-human insulin Receptor-β (CT-3)	monoclonal	mouse	Santa Cruz	1.0 µg/ml
rabbit anti-human LRP1 (H-80)	polyclonal	rabbit	Santa Cruz	0.8 µg/ml
Rabbit anti-human ZO-1	polyclonal	Rabbit	Thermo Fisher	1.0 µg/ml

mouse anti-human CD31	monoclonal	mouse	Dako	1.4 µg/ml
LC3B (D11)	monoclonal	rabbit	CST	3.6 µg/ml
anti-mouse CD107a (LAMP-1)	monoclonal	rat	BioLegend	5.0 µg/ml
rabbit IgG	NA	rabbit	Millipore	0.8 µg/ml
mouse IgG	NA	rabbit	Cedarlane Lab	0.4 µg/ml
donkey anti- mouse Cy-3 (red)	polyclonal	donkey	Jackson Immuno Lab	1.9 µg/ml
Alexa Fluor 594 donkey anti-rabbit IgG	polyclonal	donkey	BioLegend	2.0 µg/ml
Alexa Fluor 488 donkey anti-mouse IgG	polyclonal	donkey	Jackson Immuno Lab	1.7 µg/ml
DAPI (Nuclear stain)	NA	NA	Thermo Fisher	0.2 µg/ml

[Reproduced from Gali CC., *et al*, (Supplementary Table 2) with the permission from Mol Cell Neurosci (1)]

3.22 Cerebromicrovascular LC3B dot coverage quantification

Image analysis was carried out using the method previously given by yang and colleagues (205). In brief, mouse brain cryosections were fluorescently co-immunostained for LC3B and CD31, three 1mm² regions were chosen and LC3B dot coverage on CD31 positive area (microvasculature) was counted manually. Digital image acquisition and quantification were conducted using NIH imageJ software (1).

3.23 Statistical analyses

Cell culture experiments were performed in duplicates or triplicates from at least three different cell preparations, as provided in the figure legends. Two-tailed Student's unpaired t-test, one-way ANOVA followed by Dunnett's multiple comparisons test, or two-way ANOVA analysis were used to analyze differences between

groups (GraphPad Software, Inc., La Jolla, CA). $p < 0.05$ was considered as statistically significant. All data are presented as means $\pm$ SD, N refers to the number of mice in each group; n refers to the number of independent *in vitro* experiments with different pBCEC isolations performed. For BM test, statistical data analysis was performed using SPSS 24 (IBM SPSS Statistics, Vienna, Austria) and SigmaPlot 13 (Systat Software Inc, San Jose, CA, USA). Between and within subject differences were assessed by two-way repeated-measures ANOVA. Post-hoc analysis of group differences was performed by one-way repeated measures ANOVA or by two-way ANOVA followed by Tukey's post hoc comparison, as appropriate. Probability values < 0.05 were regarded as statistically significant. BM data are presented as means $\pm$ SEM, N refers to the number of mice in each group (1).

RESULTS

[Parts of this chapter have been literally published in “Gali CC, Fanaee-Danesh E, Zandi-Lang M, Albrecher NM, Tam-Amersdorfer C, Stracke A, et al. Amyloid-beta impairs insulin signaling by accelerating autophagy-lysosomal degradation of LRP-1 and IR-beta in blood-brain barrier endothelial cells in vitro and in 3XTg-AD mice. Mol Cell Neurosci. 2019 Jul 2;99:103390.”, with the permission of reuse from Mol Cell Neurosci]

4.1 Part 1: Impact of 3XTg-AD transgenes and HFD feeding on cerebral A β accumulation, memory, and peripheral and central insulin signaling

In this thesis, 3XTg-AD mice procured from The Jackson Laboratory were used to investigate the *in vivo* effects of A β burden on central and cerebral insulin signaling. 3XTg-AD mice display progressive increase in A β burden, with intracellular A β reactivity at the age as early as 3-4 months. Impairment in synaptic conductivity and long-term potentiation becomes evident by the age of 6 months, followed by detection of hyperphosphorylated tau aggregates between 12-15 months. This mouse model exhibits pathological A β plaque and neurofibrillary tangle pathology, traits similar to human AD pathology (The Jackson Laboratory) (200).

4.1.1 Genotyping of 3XTg-AD mice carrying mutations for human APP_{swe}, PS1_{M146V} and tau_{P301L}

3XTg-AD mice were generated by microinjecting human APP_{swe} and human microtubule associated protein tau (tau_{P301L}) constructs into single-cell embryos of PS1_{M146V} homozygous mice (200). Both the human APP_{swe} and tau_{P301L} constructs are integrated into the same locus of the chromosome and are under the control of mouse Thy1.2 regulatory element. 3XTg-AD homozygous mice show ~6 and ~7 fold higher levels of human APP_{swe} and tau_{P301L}, compared to NTg mice (The Jackson Laboratory) (200). Human APP (marker for APP_{swe} and tau_{P301L}) and PS1_{M146V} gene expression was confirmed by analyzing genomic DNA collected from tail tips of 3XTg-AD mice, as described in materials and methods section 3.8 (Fig. 16A&B).

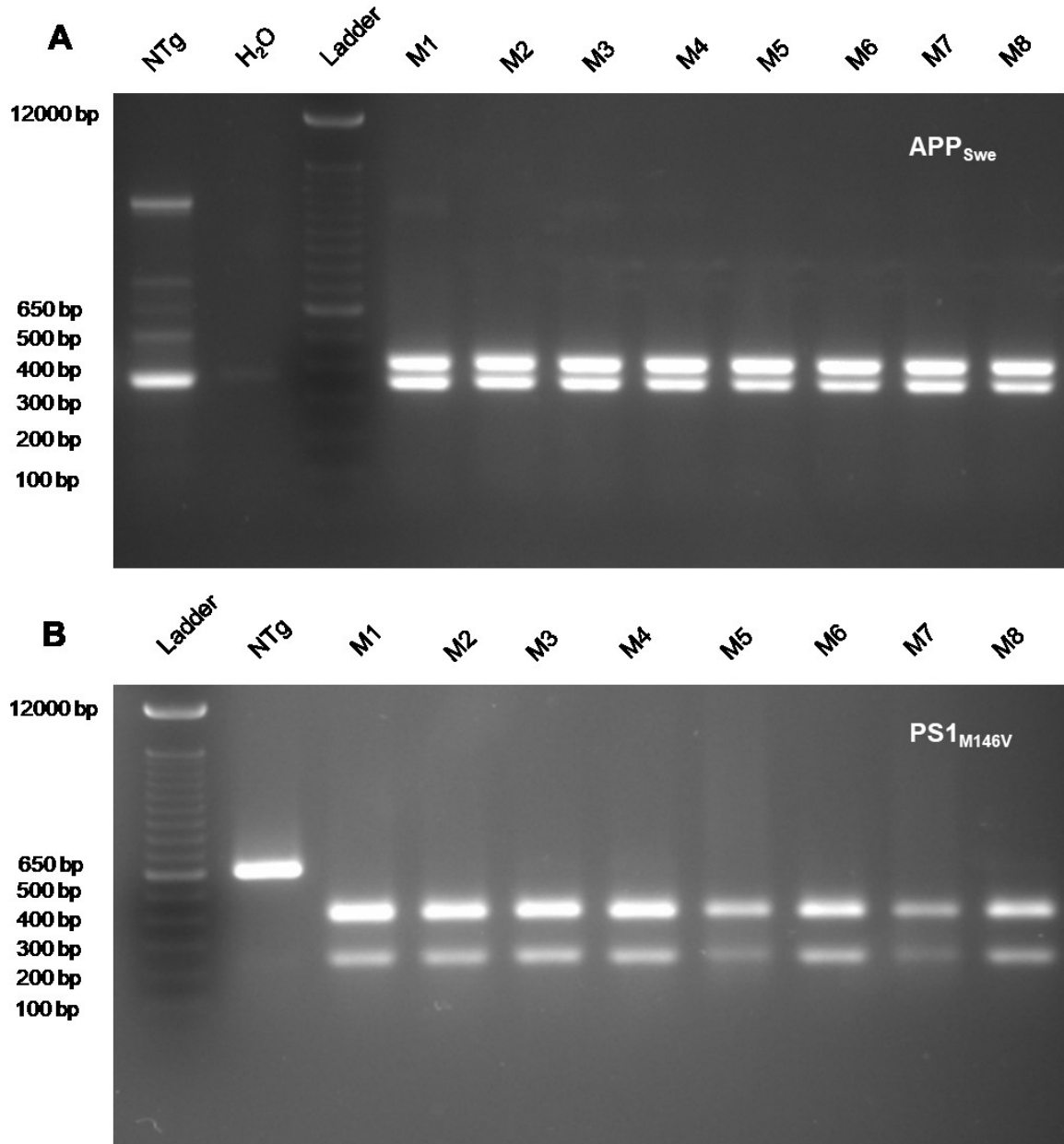


Figure 16: Confirmation of mutations for human APP_{Swe} and PS1_{M146V} genes in 3XTg-AD mice
 (A) Representative gel image showing genotyping of 3XTg-AD mice expressing APP_{Swe} (lanes M1-M8), detected at 377 bp, compared to NTg control (lane 1) and H₂O control (lane 2). (B) Representative gel image of separated PCR products showing PS1_{M146V} transgene expression at 350 bp and 180 bp, amplified in 3XTg-AD mice (lanes M1-M8), absent in NTg mice (lane 2).

4.1.2 HFD feeding enhanced body weight gain and worsened glucose tolerance in NTg and 3XTg-AD mice

As anticipated, HFD feeding for 23 weeks significantly enhanced the body weight (BW) in male and female mice, independent of genotype and sex, in NTg

mice (male: $F_{20,377}=20.13$, $p<0.0001$ and female: $F_{20,355}=1.78$, $p<0.0221$) and in 3XTg-AD mice (male: $F_{20,245}=3.15$, $p<0.0001$ and female: $F_{20,261}=3.92$, $p<0.0001$) vs chow fed mice (**Fig. 17A**). Interestingly, gender and genotype distinctions were apparent in HFD fed mice. Female NTg mice and male 3XTg-AD mice gained significantly less BW when compared with corresponding HFD fed 3XTg-AD female and NTg male mice (**Fig. 17A**); these changes in BW corresponded to diet intake over the period of the feeding study (**Fig. 17C**). Interestingly, higher food intake was observed in female 3XTg-AD, resulting in higher BW gain ($87\pm 20.1\%$ vs chow fed 3XTg-AD mice; $F_{20,261}=3.92$, $p<0.0001$) than female NTg mice ($39\pm 7.7\%$ vs NTg chow fed mice; $F_{20,355}=1.77$, $p<0.022$) (**Fig 17C**). HFD consumption and BW gain correlated with results of GTT. HFD worsened glucose tolerance in male and female mice, independent to sex and genotype, NTg (male: $F_{5,102}=8.02$, $p<0.0001$ and female: $F_{5,108}=6.55$, $p<0.0001$) and 3XTg-AD (male: $F_{5,65}=3.287$, $p<0.05$ and female: $F_{5,84}=3.92$, $p<0.0001$) mice vs chow fed mice (**Fig. 17B**). Interestingly, even though female NTg mice consumed less HFD and gained less BW when compared to 3XTg-AD female mice, they developed strong glucose intolerance, as indicated by elevated plasma glucose levels in GTT (**Fig. 17B**). No AD transgene associated changes in BW gain and glucose intolerance were apparent in 3XTg-AD mice. In conclusion, HFD feeding showed prominent effects on BW gain (**Fig. 17A**) and worsened glucose tolerance in both 3XTg-AD and NTg mice, independent of sex and genotype (**Fig. 17B**).

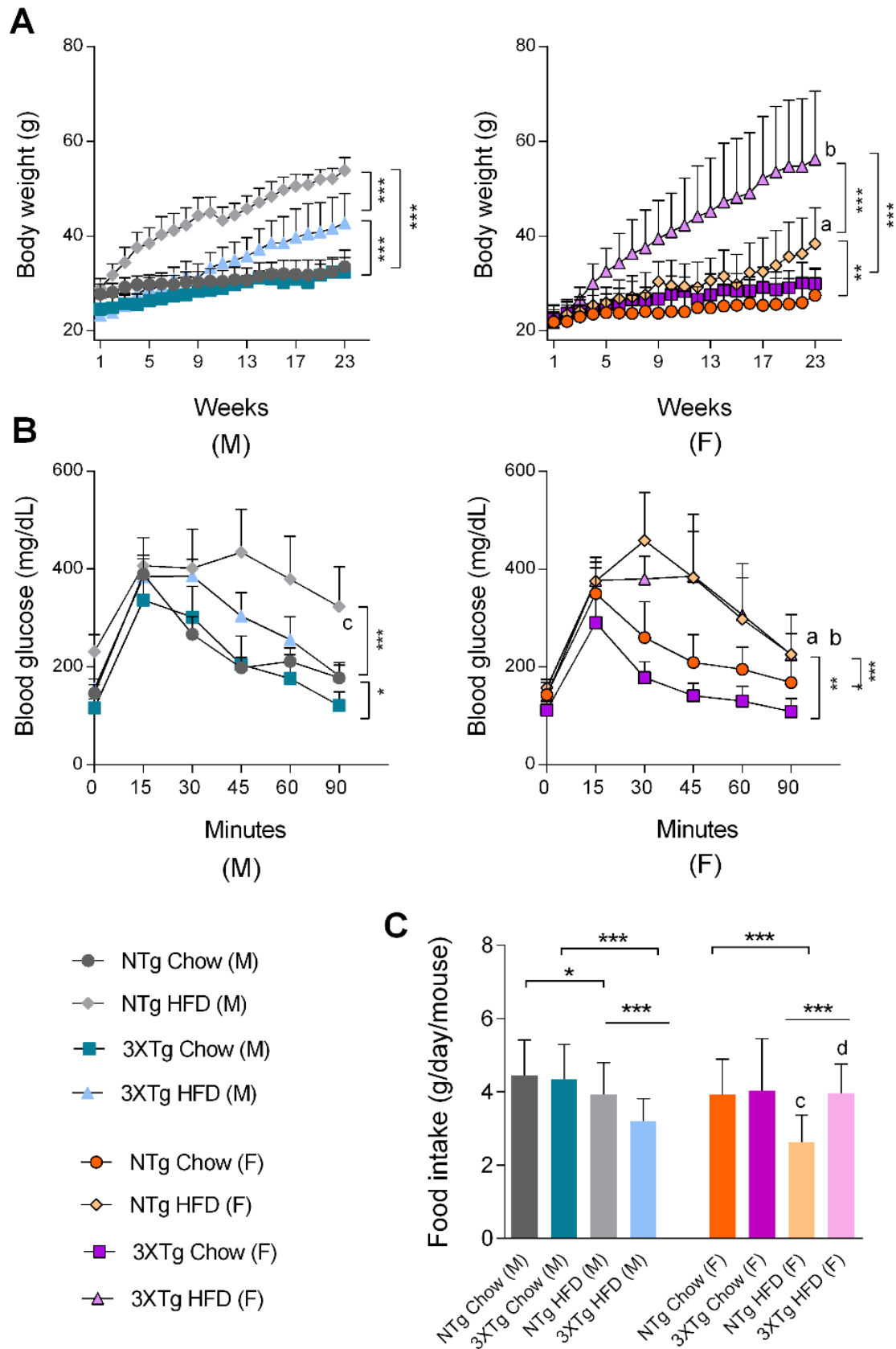


Figure 17: Impact of HFD feeding on BW and glucose tolerance in male and female 3XTg-AD and NTg mice.

(A) Weekly BW changes in male and female 3XTg-AD and NTg mice during the feeding study. Data represent mean $\pm$ SD; (N=7-10 mice/group/sex). Two-way ANOVA was performed, ** $p<0.01$ and *** $p<0.001$. Comparison between NTg HFD (M) vs NTg HFD (F) (^a $p<0.001$) and NTg HFD (F) vs 3XTg-AD HFD (F) (^b $p<0.001$). (B) Oral glucose tolerance test was performed in male and female chow and HFD fed 3XTg-AD and NTg mice at 13 weeks after initiation of feeding. Data represent mean $\pm$ SD; (N=7-10 mice/group/sex). Two-way ANOVA was performed, * $p<0.05$ and *** $p<0.001$. Comparison between NTg HFD (M) vs NTg HFD (F) (^a $p<0.05$), 3XTg-AD chow (M) vs 3XTg-AD chow (F) (^b $p<0.05$) and NTg HFD (M) vs 3XTg-AD HFD (M) (^c $p<0.05$). (C) Measurements of mean food intake by mice during 23 weeks of study. Differences between the groups were evaluated using two-tailed, unpaired students t-test. Comparison between the sexes was performed using two-tailed, unpaired students t-test (NTg HFD (M) vs NTg HFD (F); ^c $p<0.001$) and (3XTg-AD HFD (M) vs 3XTg-AD HFD (F); ^d $p<0.001$). [Reproduced from Gali CC., *et al*, (Fig. 1A-C) with the permission from Mol Cell Neurosci (1)]

4.1.3 AD transgenes and HFD feeding modulate plasma insulin and lipid profile in 3XTg-AD and NTg mice.

Plasma insulin levels and lipid parameters were quantified in chow and HFD fed, male and female, 3XTg-AD and NTg mice, as described in materials and methods sections 3.14 and 3.15. HFD feeding significantly elevated plasma insulin levels in NTg mice, a major characteristic of insulin resistance, which correlated with the worsened glucose tolerance observed (**Fig. 18A**). Gender distinctions in fasting plasma insulin levels in male and female mice were also observed. Female mice showed significantly lower fasting plasma insulin levels when compared to their male counterparts independent of transgene (**Fig. 18A**). Interestingly however, HFD feeding in 3XTg-AD mice did not show an increase in plasma insulin levels (**Fig. 18A**). These observations are in compliance with previously reported findings in glucose stimulated 3XTg-AD mice which showed pancreatic A β deposition, beta cell atrophy and low insulin release from pancreatic beta cells to plasma (206, 207). 3XTg-AD mice demonstrated significantly elevated plasma NEFA and TG levels, regardless of the feeding condition (**Fig. 18B&C**). Chow fed female but not male 3XTg-AD mice demonstrated elevated plasma cholesterol levels when compared to their chow fed NTg counterparts, indicating a metabolic shift induced by AD transgenes in female 3XTg-AD mice, which is sex specific (**Fig. 18D**). Not surprisingly however, HFD feeding significantly elevated plasma cholesterol levels in mice, independent of genotype and sex (**Fig. 18D**).

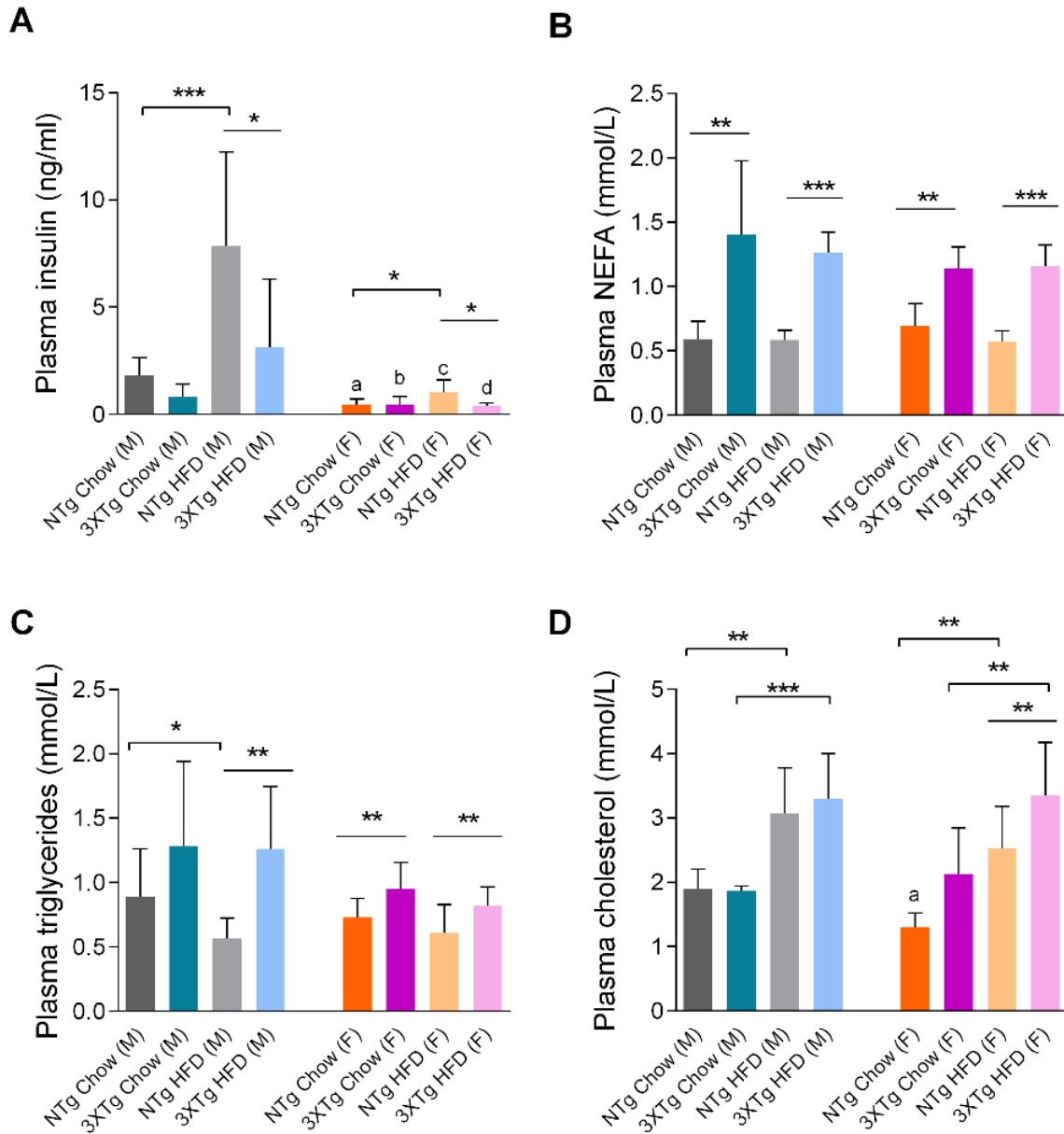


Figure 18: Impact of HFD feeding on plasma lipids and insulin levels in 3XTg-AD and NTg mice.

Plasma was isolated at the end of the feeding study and was assayed for (A) Insulin; (B) NEFA; (C) Triglycerides; (D) Cholesterol. Data represent mean $\pm$ SD; (N=7-9 mice/group, except for the 3XTg-AD male chow fed group, where N=4 mice). Differences between the groups were evaluated using two-tailed, unpaired students t-test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Comparison between the sexes was performed using two-tailed, unpaired students t-test (NTg chow (M) vs NTg chow (F); ^a $p < 0.001$), (3XTg-AD chow (M) vs 3XTg-AD chow (F); ^b $p < 0.05$), (NTg HFD (M) vs NTg HFD (F); ^c $p < 0.001$) and (3XTg-AD HFD (M) vs 3XTg-AD HFD (F); ^d $p < 0.001$). [Reproduced from Gali CC., *et al*, (Fig. 1D-G) with the permission from Mol Cell Neurosci (1)]

4.1.4 HFD feeding aggravates A β burden in 3XTg-AD mouse brain

To understand the impact of AD transgenes and HFD feeding on brain A β burden in 3XTg-AD mice, we extracted soluble and insoluble A β fractions from 3XTg-AD and NTg mouse brains, and performed A β ₁₋₄₀ and A β ₁₋₄₂ ELISA, respectively, as described in materials and methods sections **3.16** and **3.17**. No traceable amounts of soluble and insoluble A β was detected in DEA and FA brain fractions of NTg mice by ELISA assay (data not shown). Soluble and insoluble brain A β fractions from 3XTg-AD mice showed detectable A β ₁₋₄₀ and A β ₁₋₄₂ peptides (**Fig. 19A&B**). Moreover, feeding HFD significantly elevated brain A β ₁₋₄₀ and A β ₁₋₄₂ levels in both male and female 3XTg-AD mice. Importantly, female 3XTg-AD showed higher levels of A β ₁₋₄₀ and A β ₁₋₄₂ in soluble and insoluble fractions when compared to age matched male mice, indicating a sex specific influence of AD transgenes on brain A β production (**Fig. 19A&B**). Similarly, immunofluorescence microscopy revealed strong 6E10 immunoreactivity in hippocampi of 3XTg-AD mice, but not in those of NTg mice (**Fig. 19C**).

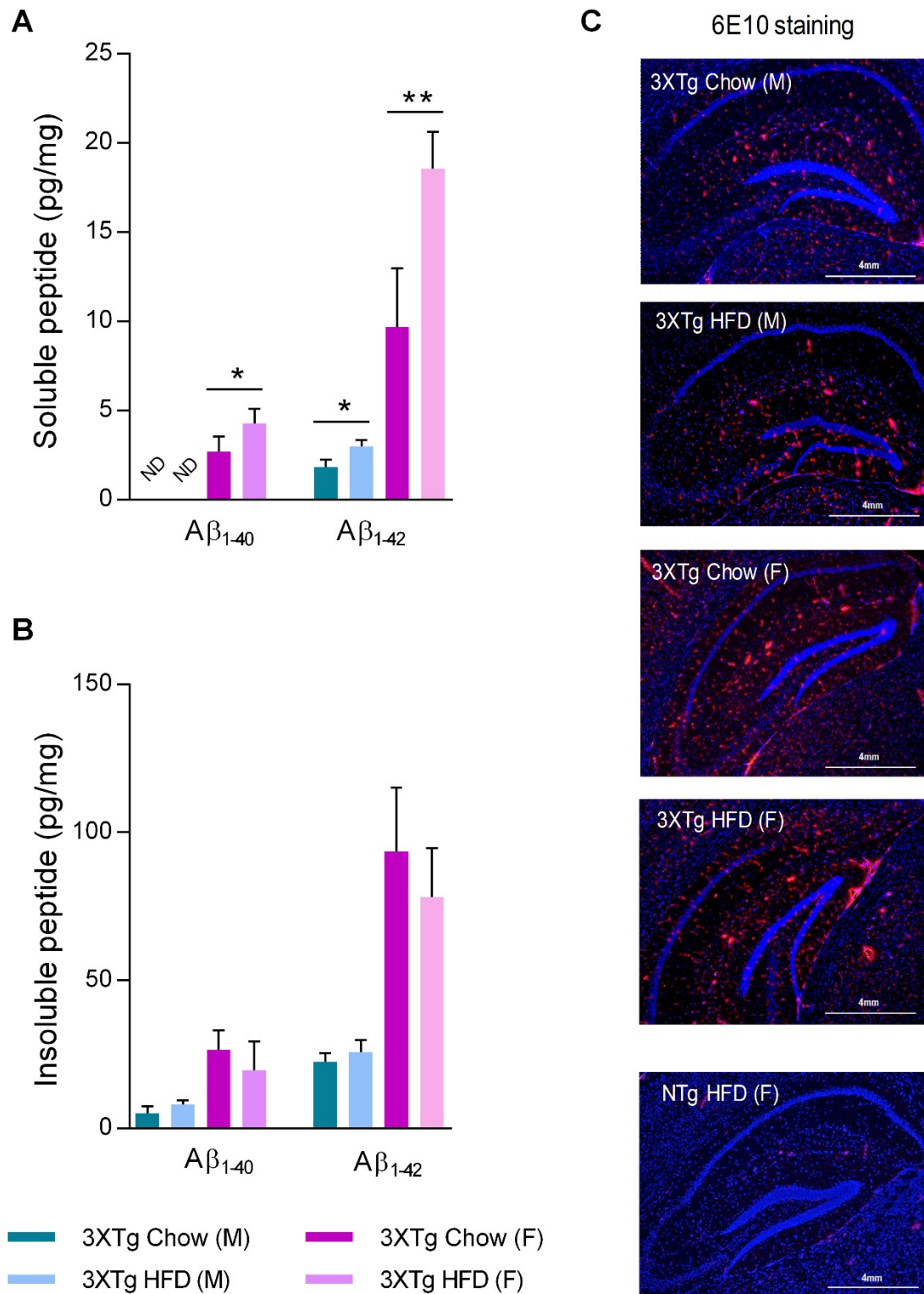


Figure 19: Effects of AD transgene, gender and HFD feeding on cerebral A β burden.

Diethylamine soluble (soluble) and formic acid soluble (insoluble) A β was extracted from the cortices of 3XTg-AD mice. (A) Soluble and (B) insoluble human A β_{1-40} and A β_{1-42} levels in total brain paren-

chyma of chow and HFD-fed 3XTg-AD mice, quantified by ELISA (N=4 mice per group). Data represent means $\pm$ SD. Statistical significance was determined using two-tailed, unpaired students t-test (* p <0.05 and ** p <0.01). ND, non-detectable. C) Representative IF staining of APP/A β burden in the hippocampus of chow and HFD fed 3XTg-AD and NTg mice. DAPI staining in blue, 6E10 staining in red. [Reproduced from Gali CC., *et al*, (Fig. 2A-C) with the permission from Mol Cell Neurosci (1)]

4.1.5 Effect of HFD feeding on memory impairment in male and female 3XTg-AD mice

To understand the effect of AD transgenes and HFD feeding on memory in 9-month-old 3XTg-AD and NTg mice, we used BM study to assess the anxiety and memory in mice (as described in materials and methods section 3.11). Behavioral tests were performed at the end of the 20th week of HFD feeding. In this study, first-hole latency indicates anxiety and target-hole latency indicates cognitive performance (**Fig. 20A-D**). BM analysis showed that 3XTg-AD female mice performed worse in finding the target hole and first hole, during the acquisition trial (**Fig. 20A&B**). Furthermore, HFD feeding worsened first-hole latency in female 3XTg-AD mice (**Fig. 20A&B**). Interestingly, HFD feeding or AD transgenes has no effects on memory in male mice. These changes in memory correlate with brain A β burden in male and female 3XTg-AD mice (**Fig. 19A&B**). Two-way RM ANOVA for the latency to find the target hole revealed that the learning performance of female mice over multiple trials depended on both the genotype and the diet (**Fig. 20A&B**). In detail, between subject analysis disclosed that female 3XTg-AD mice performed overall worse than their corresponding NTg mice (genotype main effect: $F_{1,27}=20.161$; $P<0.001$) indicating a learning deficit (**Fig. 17A**). When looking at performance over time (within subject analysis), statistics showed that the animals get faster in finding the target hole upon repeated trials (trial main effect: $F_{5,135}=5.401$; $P<0.001$) depending on the diet (trial x diet interaction: $F_{5,135}=3.088$; $P=0.011$). Post-hoc analysis disclosed that control diet animals did not get faster over multiple trials independently of the genotype, but animals on HFD did ($F_{5,65}=5.503$; $P<0.001$). In assessing this finding, we performed two-way ANOVAs for each time point (trial 1-6). For trial 1 this analysis disclosed a significant diet x genotype interaction ($F_{1,27}=6.308$; $P=0.018$). Posthoc testing showed that 3XTg-AD mice on HFD performed significantly worse during the first trial than both 3XTg-AD on control diet and NTg mice on HFD, indicating a negative impact of HFD exclusively on female

3XTg-AD mice, but not on female NTg mice (**Fig. 20A**). Closer analysis of the other trials revealed no significant genotype x diet interaction, but significant genotype main effects for trial 2 ($F_{1,27}=6.770$; $P=0.015$), trial 3 ($F_{1,27}=10.463$; $P=0.003$) and trial 6 ($F_{1,27}=5.854$; $P=0.023$), suggesting a learning deficit of the 3XTg-AD mice independent of the diet. The number of primary errors did not differ overall between the subjects and also not on any given trial (trial 1-6) except for a transient diet effect on trial 4 (diet main effect: $F_{1,27}=5.567$; $P=0.026$). However, all animals made less errors over time (trial main effect: $F_{5,135}=3.676$; $P=0.004$) (**Fig. 20A**).

First hole latency was monitored to assess the anxiety in mice, Two-way RM ANOVA showed a significant genotype x diet interaction between subjects for this parameter ($F_{1,27}=8.166$; $P=0.008$). Posthoc analysis revealed that overall female 3XTg-AD HFD fed mice needed significantly more time to approach any hole during the test sessions than 3XTg-AD chow fed animals and NTg HFD fed mice, indicating another selective negative effect of HFD on the female 3XTg-AD mice but not on the NTg mice (**Fig. 20B**). This finding was confirmed by analysis of the individual trials. Specifically, two-way ANOVA revealed a significant diet x genotype interaction for trial 1 ($F_{1,27}=7.463$; $P=0.011$), trial 2 ($F_{1,27}=7.287$; $P=0.012$) and trial 4 ($F_{1,27}=11.002$; $P=0.003$). In all cases, posthoc testing suggested that female 3XTg-AD HFD fed mice were slower in approaching holes than 3XTg-AD chow diet and NTg HFD fed animals (**Fig. 20B**). Additionally, post-hoc analysis of two-way RM ANOVA statistics revealed that not only 3XTg-AD HFD fed mice, but also 3XTg-AD chow fed mice were slower than NTg mice in approaching the first hole during the test indicating an effect of the transgene on this parameter (**Fig. 20B**). This view is supported by results obtained in trial by trial analysis, disclosing genotype main effects for trial 3 ($F_{1,27}=5.246$; $P=0.030$), trial 5 ($F_{1,27}=9.191$; $P=0.005$) and trial 6 ($F_{1,27}=20.179$; $P<0.001$).

Similar to female mice, the number of primary errors did not differ overall between the male subjects and also not in any given trial (trials 1-6). However, also similar to female mice, all animals made fewer errors over time, which just failed to reach significance (trial main effect: $F_{3,655,160}=2.179$; $P=0.081$) (**Fig. 20C&D**).

Analysis of the first hole latency revealed that male 3XTg-AD mice independent of diet were overall slower in approaching any hole (genotype main effect:

$F_{1,32}=13.803$; $P=0.001$) (**Fig. 20D**). In line with this, trial by trial analysis showed significant genotype main effects at trial 1 ($F_{1,32}=12.055$; $P=0.002$) and trial 2 ($F_{1,32}=7.857$; $P=0.009$), indicating heightened anxiety levels at the beginning of the training phase (**Fig. 20C&D**). Within subject analysis disclosed that also male animals became faster in approaching the target hole upon repeated trials ($F_{2,467,160}=8.936$; $P<0.001$), but this depended on the genotype (trial x genotype interaction: $F_{2,467,78.953}=6.894$; $P=0.001$) (**Fig. 20C&D**).

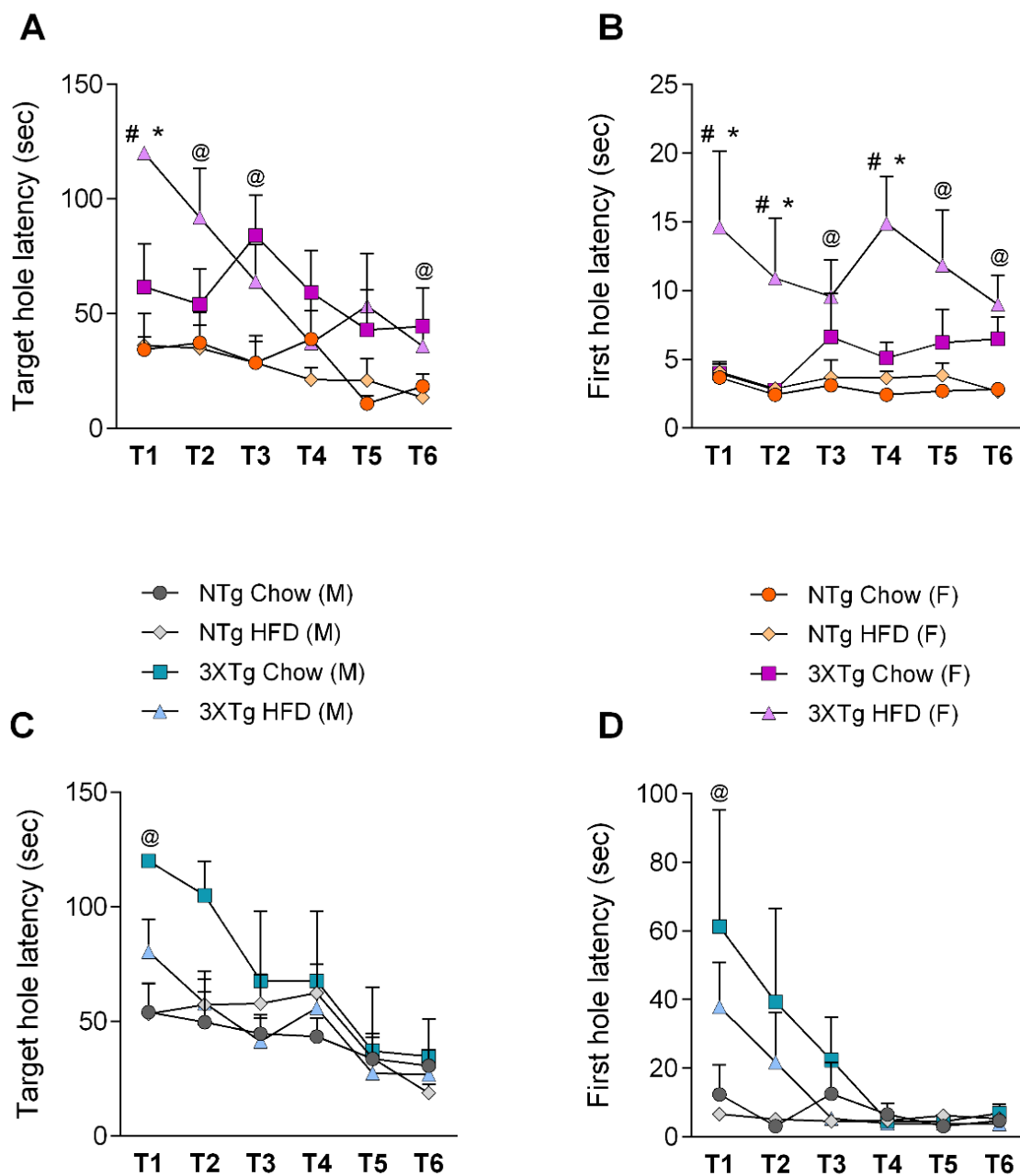


Figure 20: Effects of AD transgene, gender and HFD on anxiety and memory in mice.

Animals were tested for anxiety and memory using Barnes Maze (BM). (A, C) target hole latency, which indicates cognitive function or memory, (B, D) first hole latency indicates anxiety. N=8 mice/group, except for 3XTg-AD male chow fed, where N=4 mice/group. Data represent means $\pm$ SEM. @ $p<0.05$ for main effect: 3XTg-AD vs NTg independently of diet; * $p<0.05$ vs. NTg/chow; # $p<0.05$ vs. 3XTg-AD chow fed mice. [Reproduced from Gali CC., *et al*, (Fig. 2D) with the permission from Mol Cell Neurosci (1)]

4.1.6 Effects of HFD feeding and AD transgene on liver insulin signaling in 3XTg-AD and HFD fed mice.

Since peripheral insulin regulates A β clearance by modulating LRP-1 surface translocation and its function in liver hepatocytes (208, 209), we assessed the impact of AD transgenes and HFD feeding on liver LRP-1 and IR- β receptor levels and downstream insulin signaling (pAKT) using WB (**Fig. 21A-D**). Liver protein lysates from mice were isolated according to the methods described in the materials and methods section 3.4 and were subjected to WB (as described under the section materials and methods 3.6). Since 3XTg-AD mice showed low plasma insulin levels (**Fig. 18A**), we investigated the influence of HFD diet and AD transgenes on liver LRP-1 levels. HFD feeding significantly reduced liver LRP-1 levels in both male and female mice in both genotypes (**Fig. 21A&B**). Strikingly, male and female 3XTg-AD mice showed significantly low IR- β levels, independent of diet (**Fig. 21A&C**). Downstream pAKT levels were significantly upregulated in 3XTg-AD mice, HFD feeding significantly reduced pAKT levels in these mice (**Fig. 21A&D**). In contrast, HFD feeding led to increased hepatic pAKT levels in male NTg mice ($p<0.05$), which was not evident in female NTg mice (**Fig. 21A&D**). These results indicate the impact of AD transgenes and HFD on metabolic changes in liver insulin signaling by modulating key receptors involved in insulin signaling and A β clearance in 3XTg-AD mice.

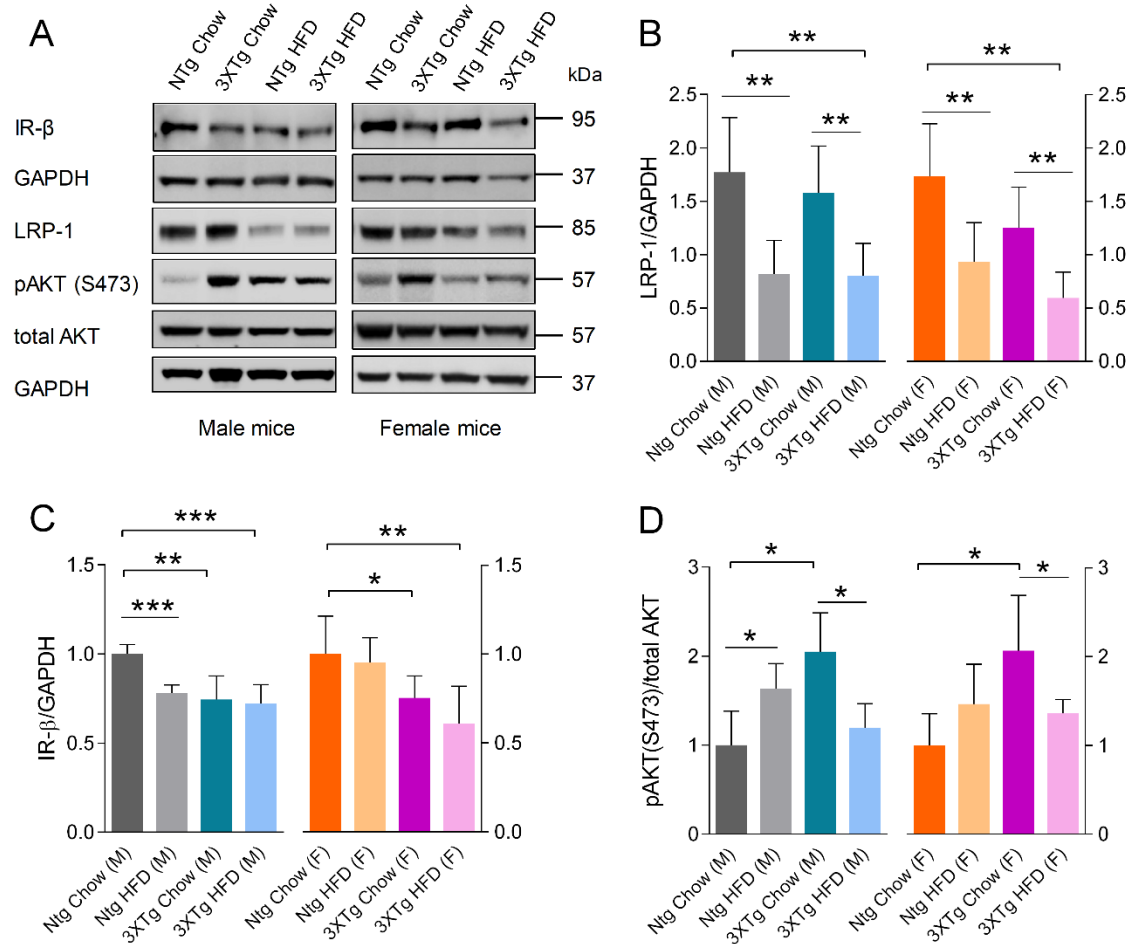


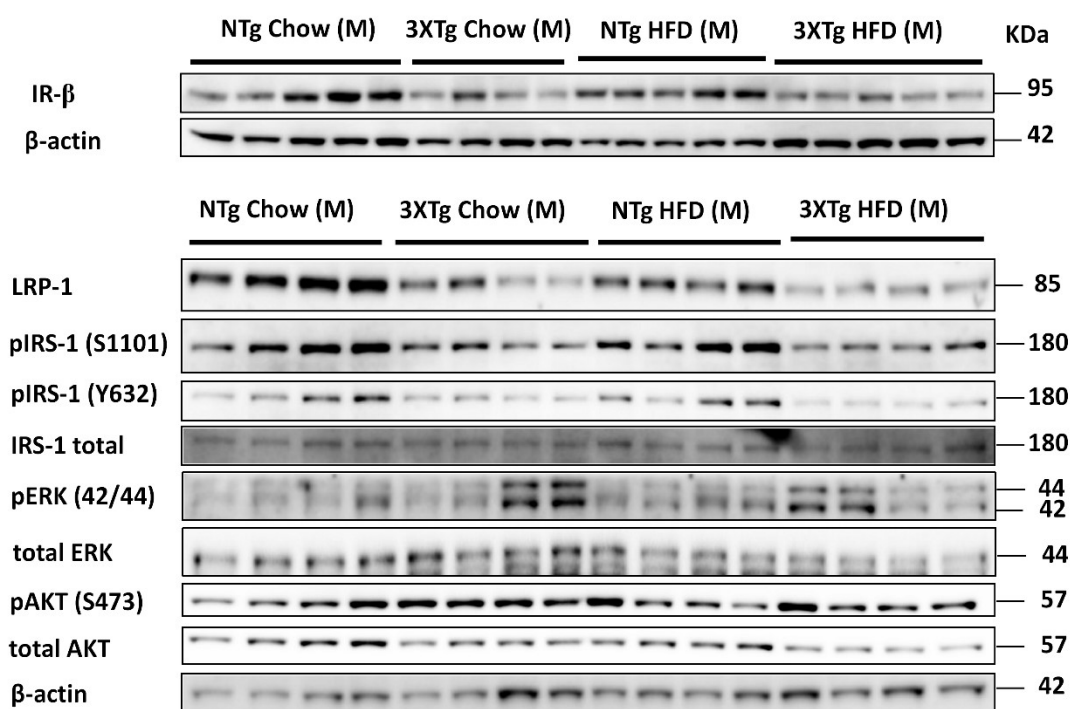
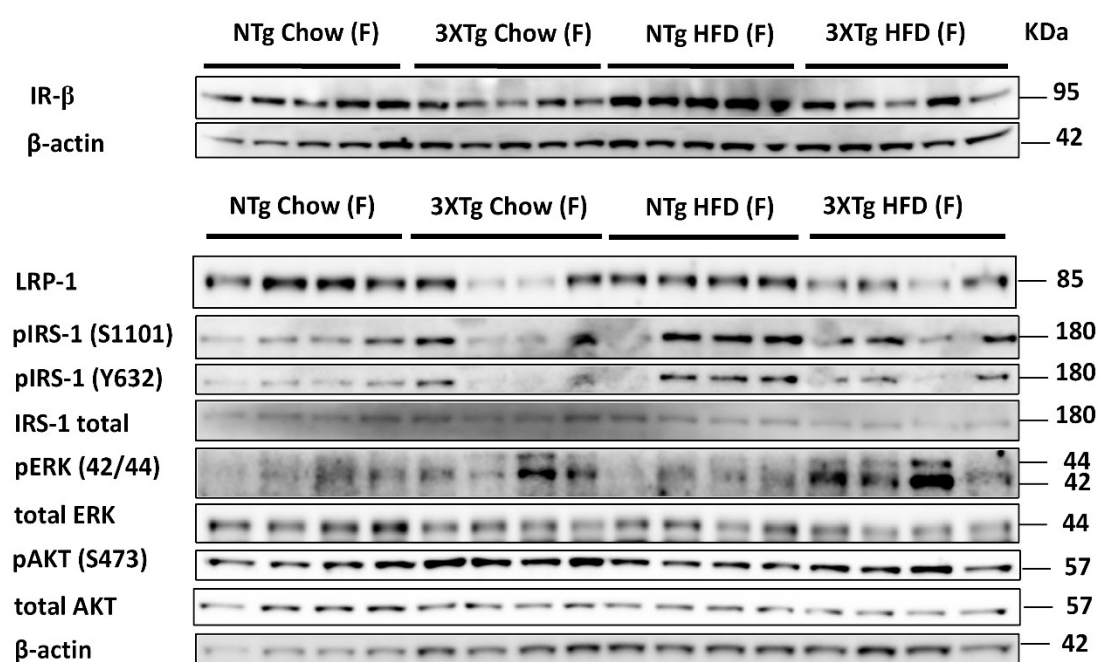
Figure 21: Effects of AD transgene, gender and HFD on liver insulin signaling and IR-β/LRP-1 levels.

Mouse liver lysates were subjected to WB using polyclonal antibodies. (A) Representative blots for IR-β, LRP-1, pAKT(s473), total AKT and GAPDH. Densitometric analysis of (B) LRP-1 (N=5) and (C) IR-β (N=5). Band intensities were normalized to GAPDH as loading control. Densitometric analysis of (D) pAKT(s473) (N=4). Intensities of pAKT(s473) bands were normalized to total AKT. Data represent mean±SD. Statistically significant differences between the groups were calculated using two-tailed, unpaired students t-test. *p<0.05 and **p<0.01. [Reproduced from Gali CC., *et al*, (Fig. 3) with the permission from Mol Cell Neurosci (1)]

4.1.7 Altered brain insulin signaling in 3XTg-AD and HFD fed mice

Brain lysates harvested from 3XTg-AD and NTg mice were subjected to WB as described in materials and methods section 3.6, and certain insulin-signaling proteins were assessed. Recently, it has been identified that neuronal LRP-1 is linked with IR-β expression and brain insulin signaling, LRP-1 disruption can lead to impaired IR-β levels and can impair insulin mediated signaling in neuronal cells (159,

160). In this thesis, significantly lower brain IR- β and LRP-1 levels were found in male and female 3XTg-AD mice (**Fig. 22A-D**), which correlates with high brain A β burden (**Fig. 22A-C**). HFD feeding further reduced LRP-1 levels in both genotypes, independent on A β burden and changes in IR- β levels (**Fig. 22C**). Further investigation of downstream insulin signaling proteins like pIRS-1(S1101) revealed that key proteins involved in insulin signaling processes were significantly downregulated in 3XTg-AD mice (**Fig. 22E**). Strikingly, pIRS-1 levels were significantly upregulated in NTg female mice fed with HFD, representing a hyper-phosphorylation state, consequently leading to downregulation of downstream insulin signaling (**Fig. 22E**). Any imbalance in changes of insulin signaling apparatus may lead to insulin resistance. Interestingly, significantly higher pAKT(S473) levels were observed in 3XTg-AD mice, this hyperactivation state of downstream insulin signaling presumably associates with “selective insulin resistance”, as described earlier; this paradoxical downstream activation state may in turn disrupt upstream insulin signaling by modulating the phosphorylation of upstream mediators like IR and receptor associated proteins (**Fig. 22F**). Similarly, significantly elevated pERK(42/44) levels were observed in 3XTg-AD mice independent of HFD feeding. HFD feeding elevated pERK levels in female NTg mice but not in male mice (**Fig. 22G**). In consummate, both upstream (pIRS-1) and downstream (pAKT and pERK) insulin signaling was altered in 3XTg-AD mice, HFD mediated changes were observed downstream (pAKT) in NTg mice and further impaired insulin signaling in 3XTg-AD mouse brain.

A**B**

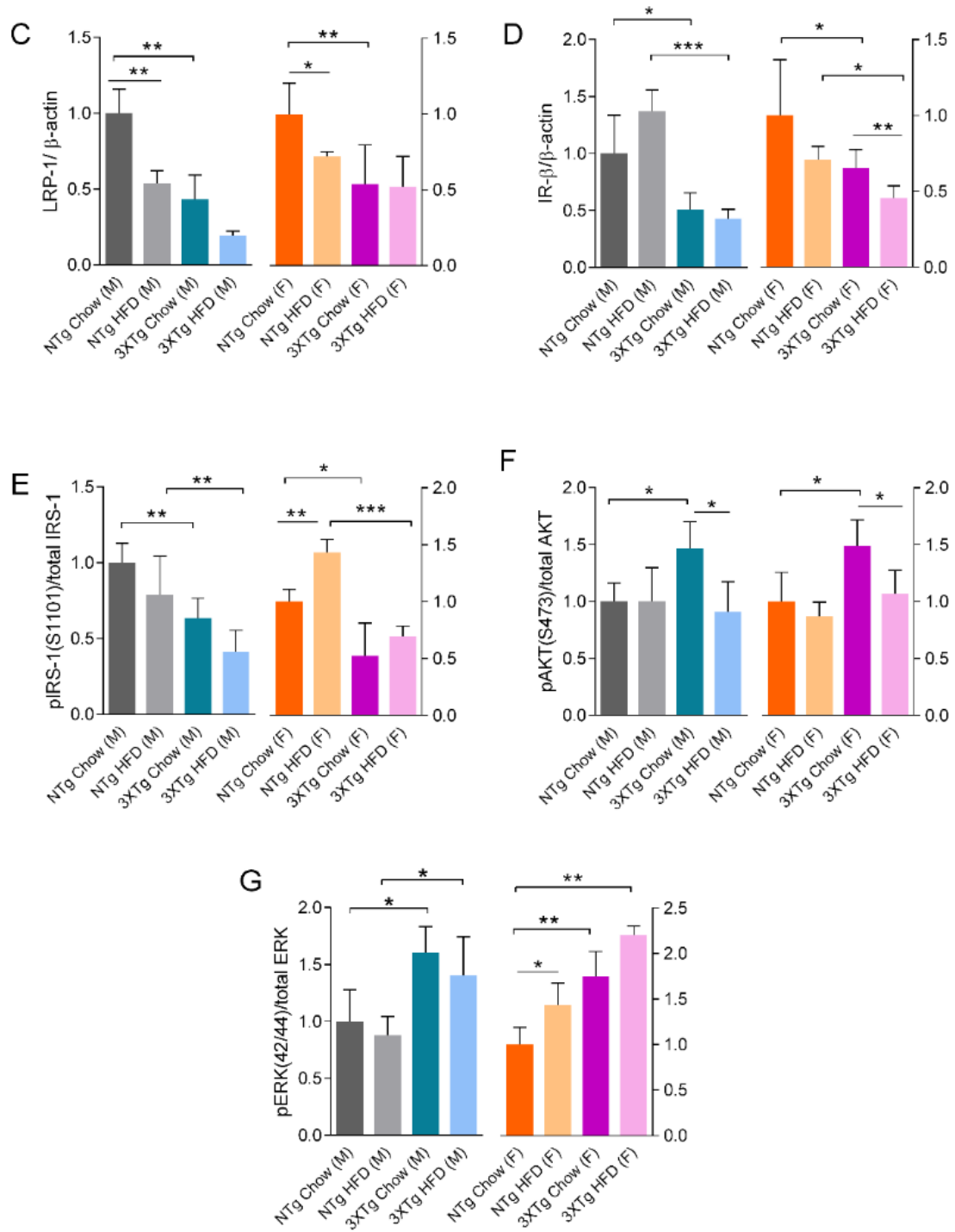


Figure 22: Modified brain insulin signaling and LRP-1 levels in 3XTg-AD and HFD fed mice.

(A,B) Representative WB for brain IR- β , LRP-1, pIRS-1(S1101), IRS-1, pERK(42/44), ERK, pAKT, total AKT and β -actin levels in male and female mice of both the genotypes. Densitometric evaluation of (C) IR- β and (D) LRP-1 (N=5) as normalized to β -actin. Densitometric analysis of (E) pIRS-1(S1101), (F) pAKT(s473) and (G) pERK (N=5), where band intensities were normalized to respective total protein band intensities. Data represent mean $\pm$ SD. statistically significant differences between groups were calculated using two-tailed, unpaired students t-test. *p<0.05, **p<0.01, and

*** $p < 0.001$. [Reproduced from Gali CC., *et al*, (Fig. 4) with the permission from Mol Cell Neurosci (1)]

4.1.8 LRP-1 and IR- β signature and their interaction in the mouse cerebrovascular endothelium

The BBB is an important rate limiting physiological barrier regulating A β and insulin trafficking between brain and blood. We isolated microvascular endothelium from mouse brain following the protocol given under the materials and methods section **3.18**. Purity of isolated cerebromicrovasculature was assessed by subjecting mRNA derived cDNA (described in materials and methods section **3.2.2**) to RT-qPCR (described in materials and methods section **3.2.3**) and analyzed for cerebrovascular signature using primers (primer list can be found under materials and methods section **3.2.3**; **table 5**) for genes typically expressed in individual neurovascular unit or brain cell populations. Platelet-derived growth factor receptor beta (PDGFR- β) and aminopeptidase N (APN=CD13) represent markers for pericytes; platelet endothelial cell adhesion molecule (PECAM1=CD31), and zonula occludens-1 (ZO1) represent endothelial cell markers; ionized calcium-binding adapter molecule 1 (IBA1), a marker for microglia; glial fibrillary acidic protein (GFAP), a marker for astrocytes, and aquaporin-4 (AQP-4), a marker for astrocytic end feet; synaptophysin (SYP) and microtubule-associated protein 2 (MAP2) are neuronal markers. Mouse brain microvasculature showed significantly higher amounts of CD31 (31.2 fold vs whole brain), confirming that major portion of isolated microvessels are significantly endothelial cells (**Fig. 23A**). Along with high endothelial expression, we also observed 3.1-fold higher aminopeptidase N (APN=CD13) levels indicating contaminating pericytes (**Fig. 23A**). Whereas, no microglia or neuronal cell contamination was observed. We next investigated the signature of LRP-1 and IR- β in mouse cerebro-microvasculature using double label IF stainings (as described in materials and methods section **3.21**. Using markers for endothelial cells (CD31 and ZO1), we identified that LRP-1 and IR- β proteins are expressed in mouse cerebrovascular endothelium and can interact at the level of BBB.

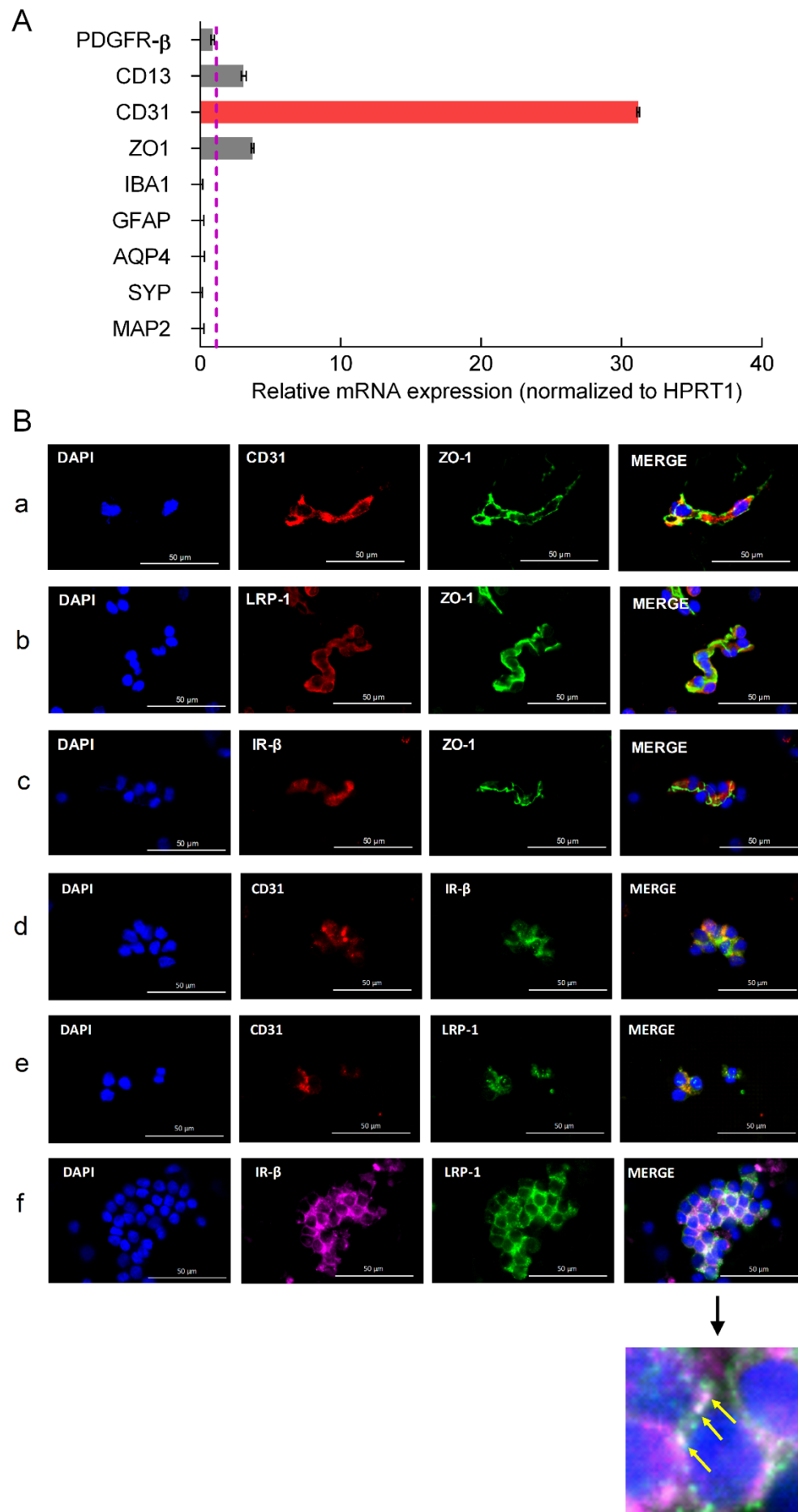


Figure 23: Purity of isolated murine brain microvasculature and double IF staining of LRP-1 and IR- β in mouse cerebromicrovascular endothelium.

(A) Purity of isolated microvessels was assessed using RT-qPCR analysis (A) CD31 enriched endothelial cells in purified mouse cerebral micro vessels. Relative mRNA expression of specific markers for endothelial cells (CD31 & ZO1), pericytes (PDGFR- β and CD13), microglia (IBA1 and GFAP), astrocytes (AQP4) and neuronal cells (SYP & MAP2), values were normalized to HPRT1. Dotted line indicates the relative gene expression of respective markers in the whole brain, also normalized to HPRT1. (B) Double IF staining was performed using markers for endothelial cells (CD31 and ZO-1) to identify the signature of low-density lipoprotein receptor-related protein 1 (LRP-1) and insulin receptor- β (IR- β), using Leica DM4000 B microscope, as shown in A. a) CD31 (red) and ZO-1 (green); b) LRP-1 (red) and ZO-1 (green); c) IR- β (red) and ZO-1 (green); d) CD31 (red) and IR- β (green); e) CD31 (red) and LRP-1 (green); f) IR- β (magenta) and LRP-1 (green). Colocalization (white) of IR- β (magenta) and LRP-1 (green) is shown using yellow arrows. [Reproduced from Gali CC., *et al*, (Fig. 5A; supplementary fig. 3&4) with the permission from Mol Cell Neurosci (1)]

4.1.9 Impaired cerebromicrovascular endothelial LRP-1 and IR- β levels in 3XTg-AD mice

Since we observed critically altered insulin signaling in brain (**Fig. 22**), we next investigated the impact of AD and HFD diet on cerebrovascular endothelial IR- β and LRP-1 protein levels, which are known to be critical in regulating insulin transport and A β trafficking at the BBB (42, 210-212). We isolated cerebromicrovasculature from the mouse brain as mentioned in materials and methods section **3.18**. Cerebromicrovascular protein lysates from 3XTg-AD and NTg mice were subjected to WB (as indicated under the materials and methods section **3.6**) to identify the changes in LRP-1 and IR- β proteins levels. We observed significantly lower LRP-1 and IR- β protein levels in male and female 3XTg-AD mice (**Fig. 24A-C**). As opposed to brain where HFD feeding impaired LRP-1 and IR- β levels (**Fig. 24C&D**), HFD feeding showed no significant effects on cerebromicrovascular endothelial LRP-1 and IR- β proteins in 3XTg-AD or NTg mice (**Fig. 24A-C**).

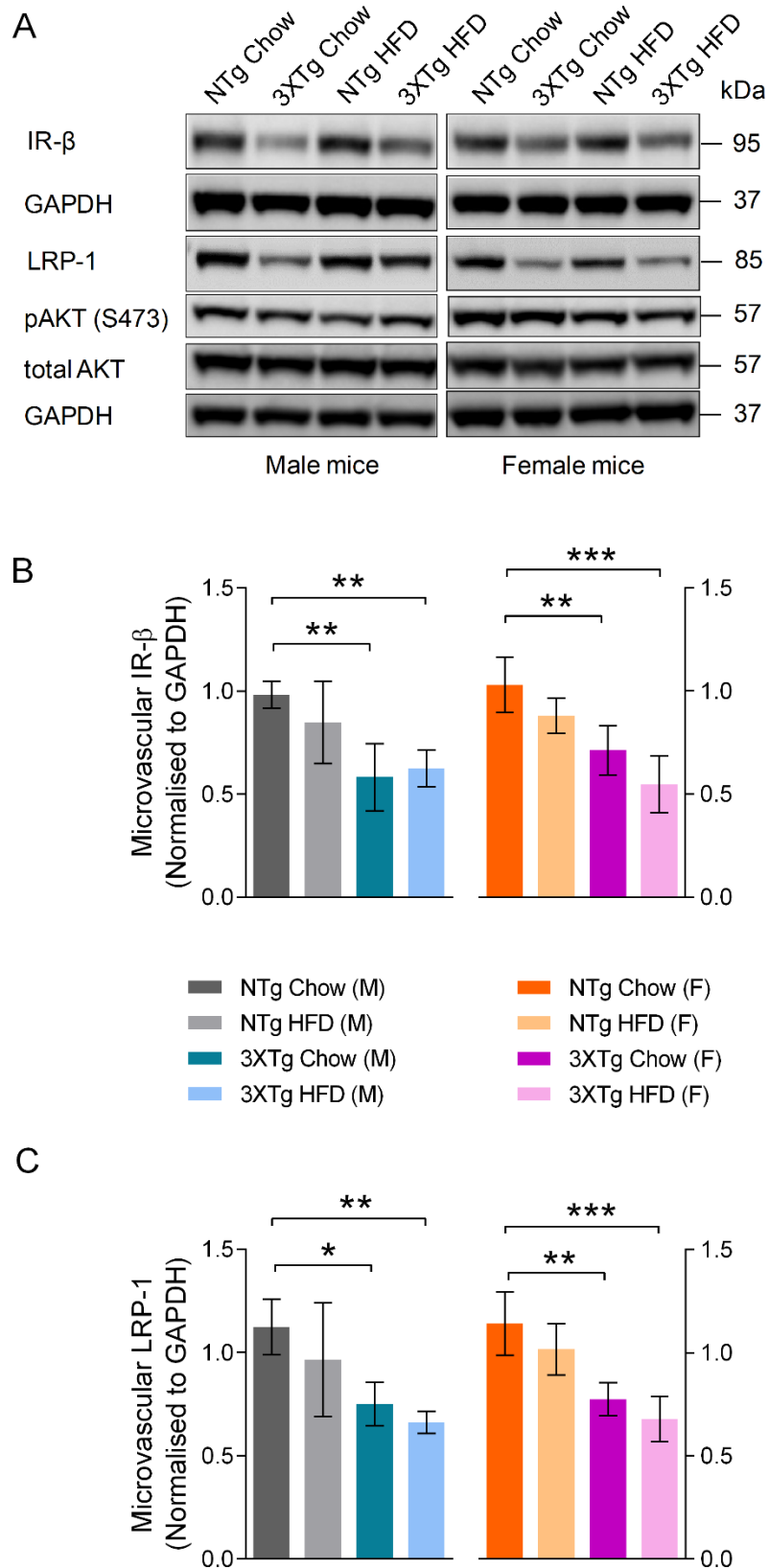


Figure 24: Effect of AD transgenes, gender and HFD on cerebrovascular endothelial LRP-1 and IR-β levels in 3XTg-AD and NTg mice.

Murine BCEC protein lysates of male and female 3XTg-AD and NTg mice of both feeding regimens were subjected to WB. (B) Representative blots for IR- β , LRP-1, pAKT, total AKT and GAPDH levels. (C & D) Densitometric analysis of specific bands for (C) IR- β and (D) LRP-1 (N=4) as normalized to GAPDH as loading control. Data represent mean $\pm$ SD; Significant differences between the groups were calculated using two-tailed, unpaired students t-test; * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. [Re-produced from Gali CC., *et al*, (Fig. 5B-D) with the permission from Mol Cell Neurosci (1)]

4.1.10 Absence of cerebromicrovascular pro-fibrillary/fibrillary A β deposits or CAA in 9-month-old male and female HFD fed 3XTg-AD mice

Since cerebromicrovascular endothelial LRP-1 and IR- β levels were down-regulated in 3XTg-AD mice (**Fig. 24A-C**) and these changes correlate with brain A β levels (**Fig. 22A&B**), we hypothesized that the changes in LRP-1 and IR- β levels may be a direct cause of A β accumulation as fibrils in cerebromicrovasculature in 3XTg-AD mice. Congo red staining was performed (materials and methods section **3.20**) to detect A β plaques and CAA using modified Puchtler's congo red staining protocol (204). As a positive control, we used 12-month-old 3XTg-AD female mice (**Fig. 25A-D**). We did not observe any fibrillary or pro-fibrillary A β aggregates in male and female 3XTg-AD mice (**Fig. 25G-H**). Hence, the changes observed in LRP-1 and IR- β levels in cerebromicrovascular endothelium in 3XTg-AD mice could be a direct effect of soluble A β produced by neuronal cells, and trafficked by BCEC.

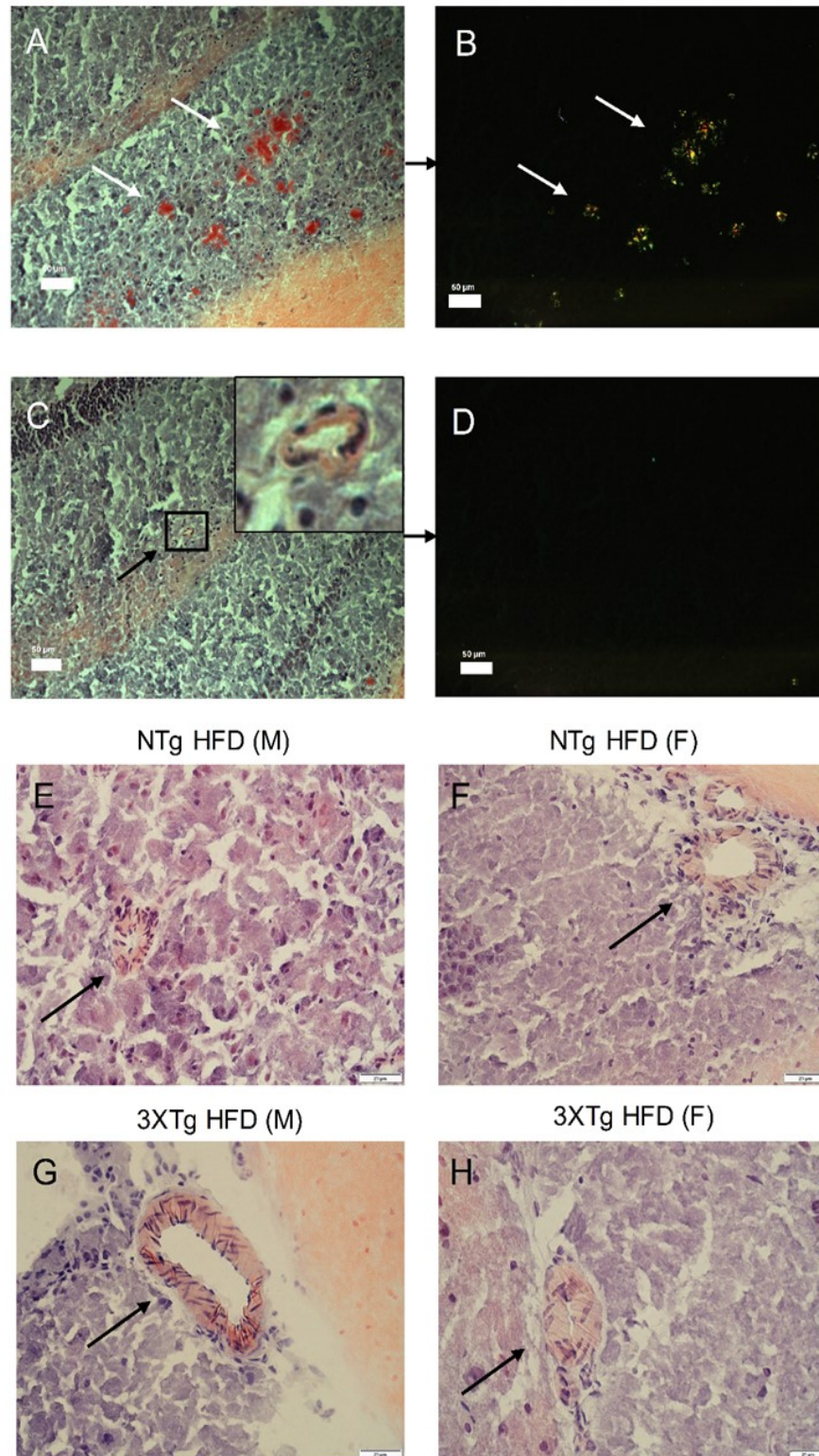


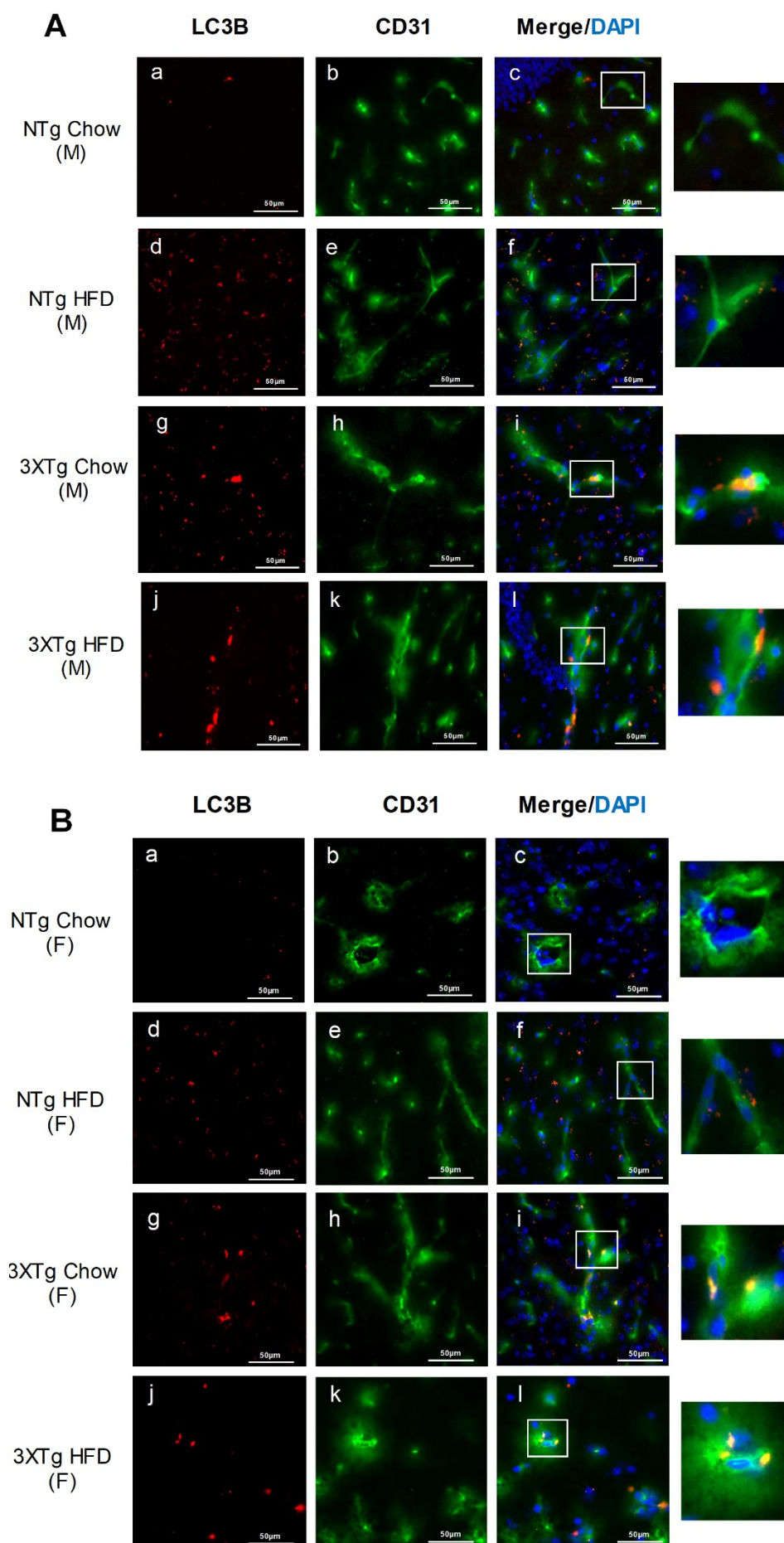
Figure 25: Effect of AD transgenes and HFD on cerebrovascular A β deposition in 3XTg-AD mice.

(A) Positive control: Puchtler's congo red staining of A β plaques (reddish-pink color) in hippocampus of 12-month-old female 3XTg-AD mice; white arrows indicate stained A β plaques. (B) Puchtler's congo red staining of A β plaques between crossed polarizers displaying apple-green bi-refringence

of section indicated in A, bi-refringence indicated with white arrows. (C) Vascular staining with Puchtler's congo red in 12-month-old female 3XTg-AD mice, absence of reddish-pink A β protofibrils in microvessel, indicated by a black arrow. (D) Absence of apple-green bi-refringence of section indicated in C. (E-H) Puchtler's congo red hippocampal cerebrovascular staining in 9-month-old male and female 3XTg-AD HFD fed mice, absence of cerebrovascular A β protofibril accumulation, black arrows indicate microvessels (hematoxylin-congo red staining). [Reproduced from Gali CC., *et al*, (Supplementary Fig. 5) with the permission from Mol Cell Neurosci (1)]

4.1.11 Elevated cerebral and cerebrovascular autophagy in 3XTg-AD mice

Autophagy-lysosomal pathways strictly regulate cell protein turnover and are responsible for surface receptor recycling. Recent studies in neuronal cell lines (114) and in the 5XFAD mouse model (115), demonstrated that A β addition or production in brain could induce autophagy, as represented by elevated LC3-II levels. We were interested to know if the changes in LRP-1 and IR- β reduction in 3XTg-AD mice correlate with elevated A β levels and autophagy. We speculated that A β trafficking to cerebrovascular endothelium in 3XTg-AD mice might influence autophagy in endothelial cells. To understand the effect of A β on cerebrovascular autophagy, we fluorescently co-immunostained mouse brain cortical sections with CD31 (endothelial marker) and LC3B (autophagy marker), and analyzed the LC3B dot coverage on CD31 positive regions (materials and methods section 3.22). We identified a significant increase in LC3B dot coverage in the cerebrovasculature of both 3XTg-AD and HFD fed mice (**Fig. 26A&B**). However, LC3B coverage on CD31 positive cells was significantly higher in male and female 3XTg-AD mice, HFD feeding further elevated these levels (**Fig. 26A&B**). The LC3B staining of CD31 positive coverage area correlates with elevated brain A β burden (**Fig. 19A&B**) and reduced cerebrovascular LRP-1 and IR- β levels in 3XTg-AD mouse brain (**Fig. 24A-C**). Next, we analyzed LC3B-II levels in whole brain lysates as a measure of autophagy in 3XTg-AD and HFD fed male and female mice. Significantly higher LC3B-II levels were observed in the brain lysates of male and female 3XTg-AD mice (**Fig. 26D&E**). Moreover, HFD feeding further elevated LC3B-II levels in both genotypes and sexes (**Fig. 26D&E**). These results indicate that autophagy induction in cerebrovasculature of 3XTg-AD mice might contribute to faster degradation of these receptors.



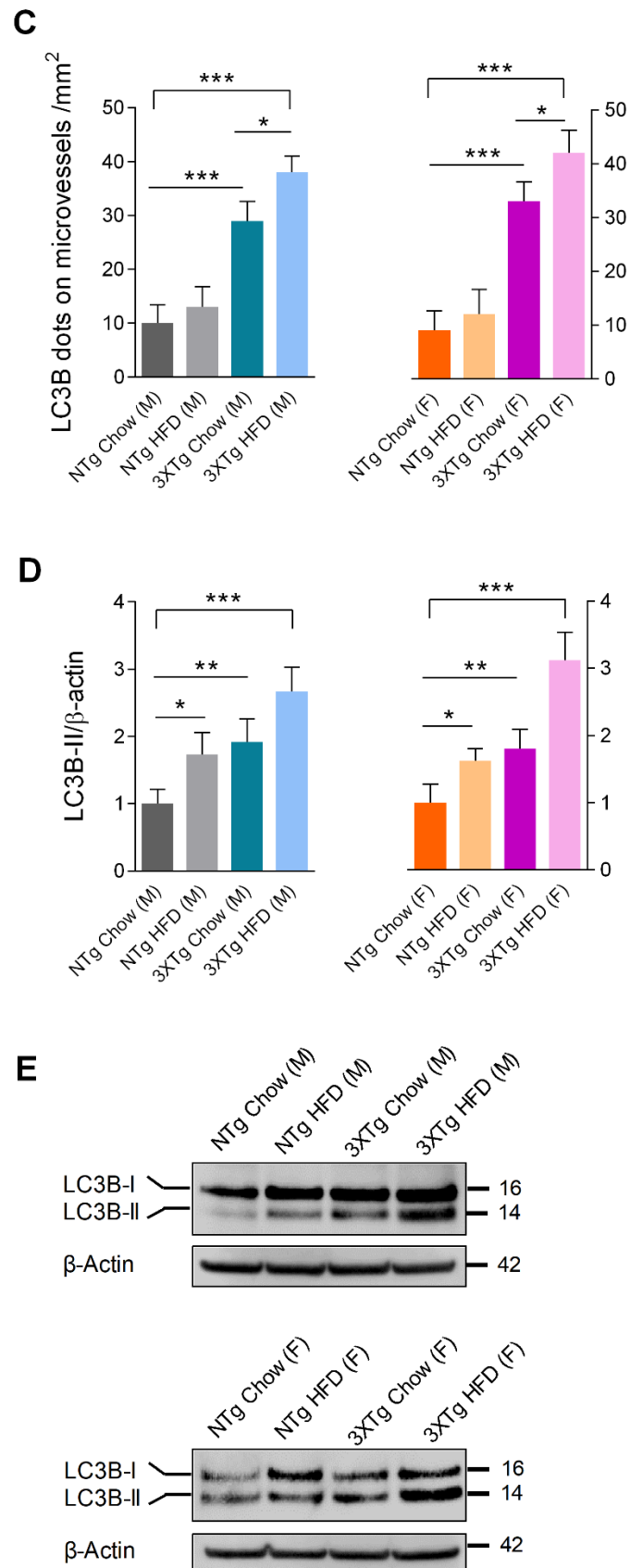


Figure 26: Elevated cerebrovascular and brain autophagy in 3XTg-AD mice.

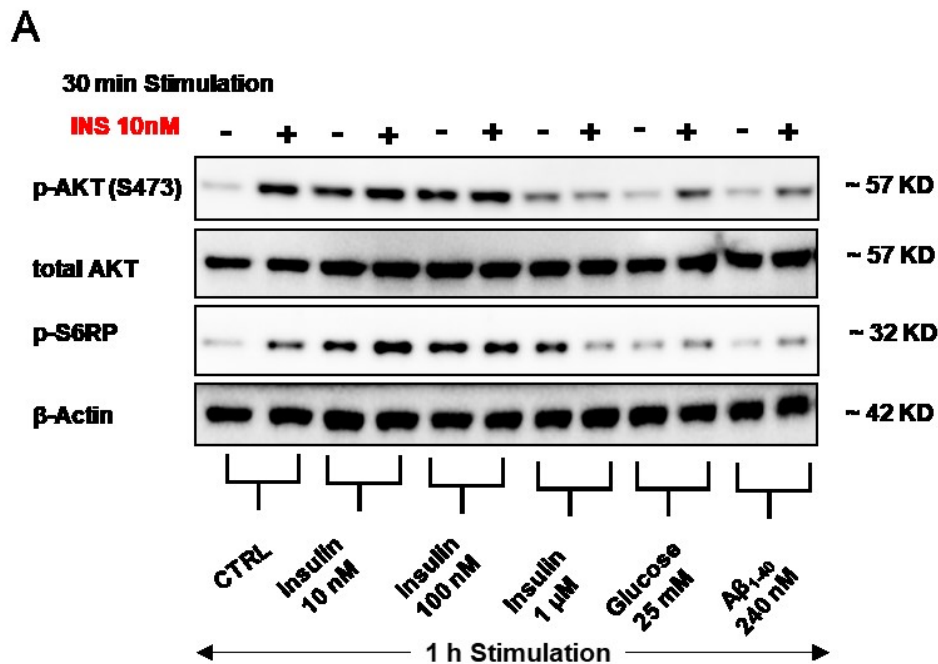
LC3B (red) and CD31 (green) double IF labeled 18 μ M brain cryosections from (A) male and (B) female HFD and chow fed mice of both genotypes, nuclear stain in blue (DAPI). White boxes indicate areas of cerebrovascular LC3B and CD31 protein colocalization (yellow). (C) Bar diagram representing quantification of LC3B dots covering the microvasculature in 1mm² fields in cortical cryosections of male and female mice of both genotypes and feeding conditions. N = 3 animals per group. Data represent mean $\pm$ SD values; one-way ANOVA was applied, followed by Dunnett's post-hoc test and intra species evaluation was done using student's unpaired t-test; *p < 0.05 and ***p < 0.001. (D) Densitometric evaluation of LC3B-II levels normalized to β -actin in brain lysates. N = 3 samples per group, Data represent mean $\pm$ SD; statistical evaluation was made using one-way ANOVA, followed by Dunnett's post-hoc test; *p < 0.05; **p < 0.01 and ***p < 0.001. (E) Representative WB for LC3B and β -actin in mouse brain lysates. [Reproduced from Gali CC., *et al*, (Fig. 6) with the permission from Mol Cell Neurosci (1)]

4.2 Part 2: Implications of A β burden on blood-brain barrier LRP-1 and IR- β regulation, and insulin signaling *in vitro*.

To address the impact of A β burden on the BBB and its effects on insulin signaling, we used primary pBCEC for *in vitro* studies.

4.2.1 Insulin signaling pathways in pBCEC

We validated active insulin signaling machinery in pBCEC upon human insulin (10 nM and 100 nM) stimulation under serum free (SF) conditions (**Fig. 27A**). High insulin (1 μ M) and glucose (25 mM), and A β_{1-40} (240 nM) treatment impaired insulin mediated signaling, as represented by reduced pAKT (S473) and downregulated phosphor-ribosomal protein S6 (pRP S6) when compared to pBCEC treated with insulin alone (**Fig. 27A**). Moreover, we confirmed that insulin activates PI3K/mTOR pathway as well as ERK pathway, represented by elevated levels of pAKT (S473) and pERK (42/44) upon insulin treatment. Insulin-signaling inhibitors such as linsitinib (Insulin-receptor tyrosine kinase inhibitor), trametinib (ERK inhibitor), NVP-BE235 (PI3K/mTOR inhibitor) and rapamycin (mTOR inhibitor) blocks insulin mediated effects by blocking these pathways in pBCEC (**Fig. 27B&C**).



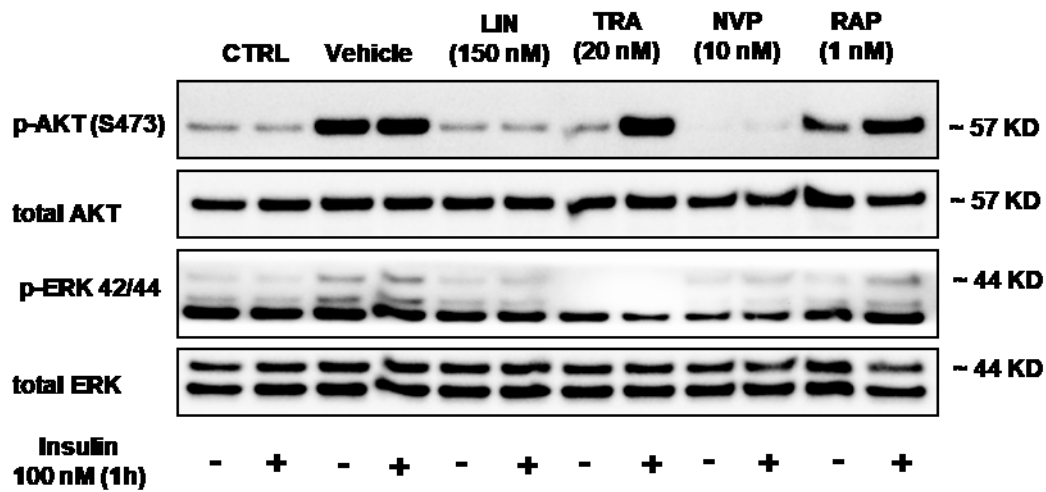
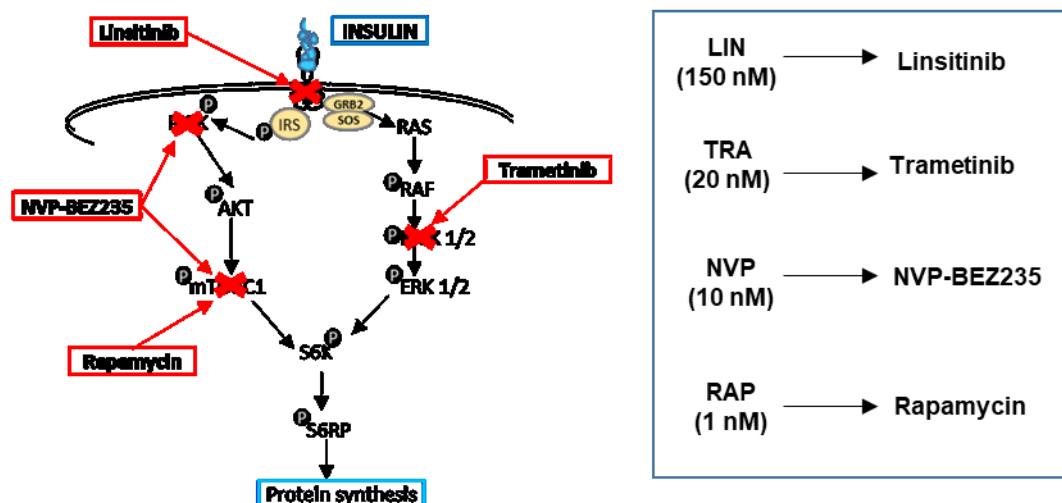
B**C**

Figure 27: Insulin signaling apparatus is conserved in pBCEC.

Serum starved pBCEC were treated with insulin (10 nM, 100 nM and 1 μ M) or glucose (25 mM) or A β ₁₋₄₀ peptide (240 nM) for 1 h, and stimulated with 10 nM insulin for 30 min in SF conditions; cell lysates were subjected to WB. (A) Representative blots for pAKT (S473), total AKT, pS6RP and β -actin. Primary pBCEC were starved for 6 h, incubated with small molecule inhibitors linsitinib (150 nM), trametinib (20 nM), NVP-BEZ235 (10 nM) and rapamycin (1 nM) for 2h and incubated with insulin (100 nM) for 1h; cells were lysed and subjected to WB. (B) Representative blots for pAKT (S473), total AKT, pERK (42/44), total ERK and β -actin. (C) Scheme demonstrating the targeting sites of small molecule inhibitors on insulin signaling.

4.2.2 A β treatment *in vitro* impairs IR- β and LRP-1 protein levels and their interaction in primary pBCEC

Since we found low IR- β and LRP-1 protein levels in the cerebromicrovascular endothelium of 3XTg-AD mice (**Fig. 24B&C**), we aimed at identifying the factors leading to these changes. To understand this phenomenon, primary pBCEC were treated with recombinant human A β peptides (A β ₁₋₄₀ and A β ₁₋₄₂; 1 μ g/ml or 240 nM) for 6 h under SF conditions. Both A β peptides significantly reduced IR- β and LRP-1 protein levels in pBCEC (**Fig. 28A&B**). Moreover, insulin induced LRP-1 levels were significantly downregulated when incubated with A β ₁₋₄₀ and A β ₁₋₄₂ peptides (**Fig. 28A&B**). More importantly, incubation with A β ₁₋₄₀ peptide for 6 h reduced IR- β and LRP-1 protein interaction in pBCEC as shown in double IF stainings (**Fig. 28C**). A β mediated changes in IR- β and LRP-1 protein levels in pBCEC were accompanied by significantly elevated levels of LC3B-I, which is primary involved in auto-phagophore formation and maturation (**Fig. 28A&B**).

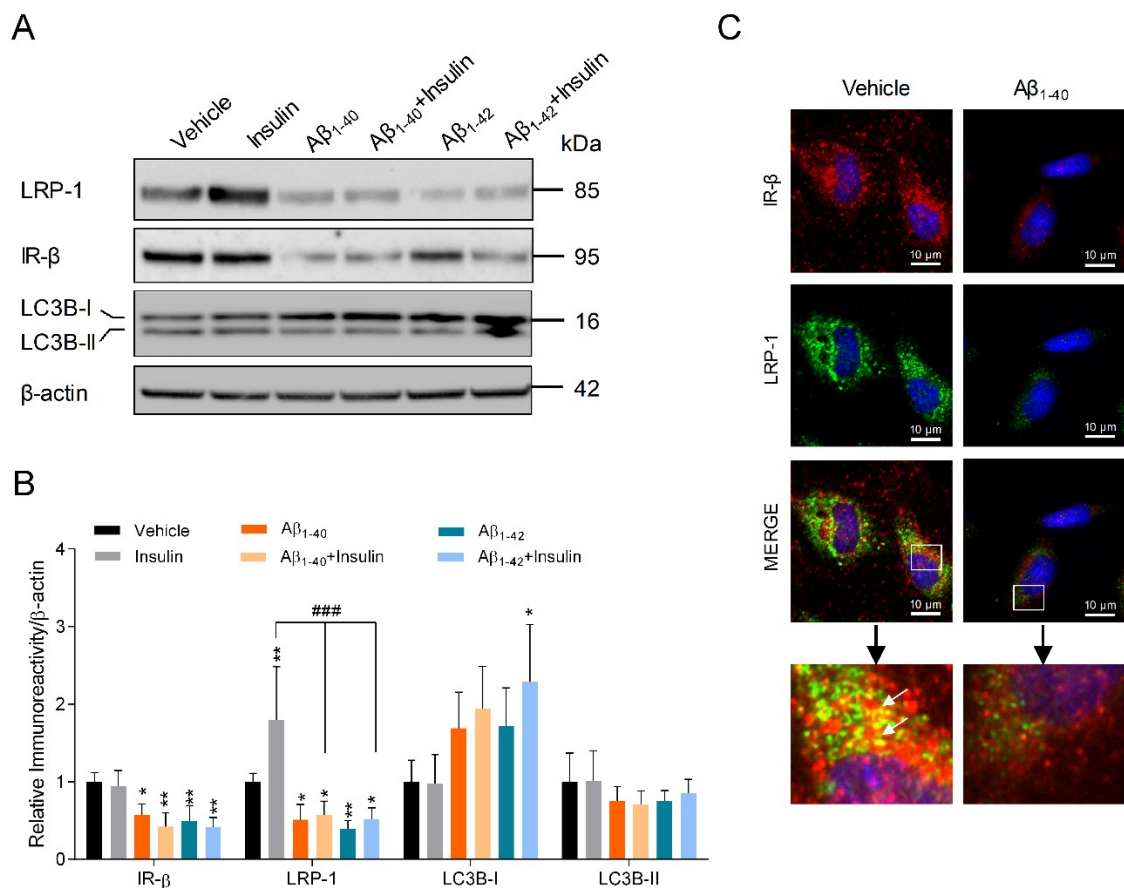


Figure 28: Effects of A β isoforms on LRP-1 and IR- β protein levels and their interaction in pBCEC.

Under SF conditions pBCEC were incubated with insulin (100 nM) with or without A β ₁₋₄₀ or A β ₁₋₄₂ peptides (240 nM) for 6 h, and cell lysates were subjected to WB. (A) Representative blots for LRP-1, IR- β , and LC3B expression. (B) Densitometric evaluation of LRP-1, IR- β , LC3B-II and LC3B-I levels. Band intensities were normalized to β -actin; n=3 experiments from different cell preparations. One-way ANOVA followed by Dunnett's post-hoc test was used. Statistically significant differences are represented as *p<0.05 and **p<0.01 vs vehicle treatment; ###p<0.001 vs insulin treatment. (C) Cells were incubated with either vehicle or A β ₁₋₄₀ peptide (240 nM) for 6 h under SF conditions, washed, fixed with 4% PFA, probed with antibodies against LRP-1 (green) and IR- β (red) to visualize the receptor interaction (yellow). [Reproduced from Gali CC., *et al*, (Fig. 7A-C) with the permission from Mol Cell Neurosci (1)]

4.2.3 A β treatment *in vitro* impairs insulin signaling in pBCEC

Both A β peptide and insulin are regarded as amyloid peptides and share similar properties (213). It is well understood that A β peptides can compete with insulin in interacting with insulin receptors and can perturb insulin mediated responses (135, 136). To test if this phenomenon occurs at the BBB level, pBCEC were incubated with A β peptides (A β ₁₋₄₀ or A β ₁₋₄₂; 240nM) with or without insulin. A significant downregulation in insulin induced pAKT(S473) activation was observed (**Fig. 29A&B**). While, pERK(42/44) levels were unaltered (**Fig. 29C**), since A β is known to induce Ras-ERK signaling (214). These findings demonstrate that A β trafficking in pBCEC can alter insulin signaling and might lead to conditions similar to insulin resistance.

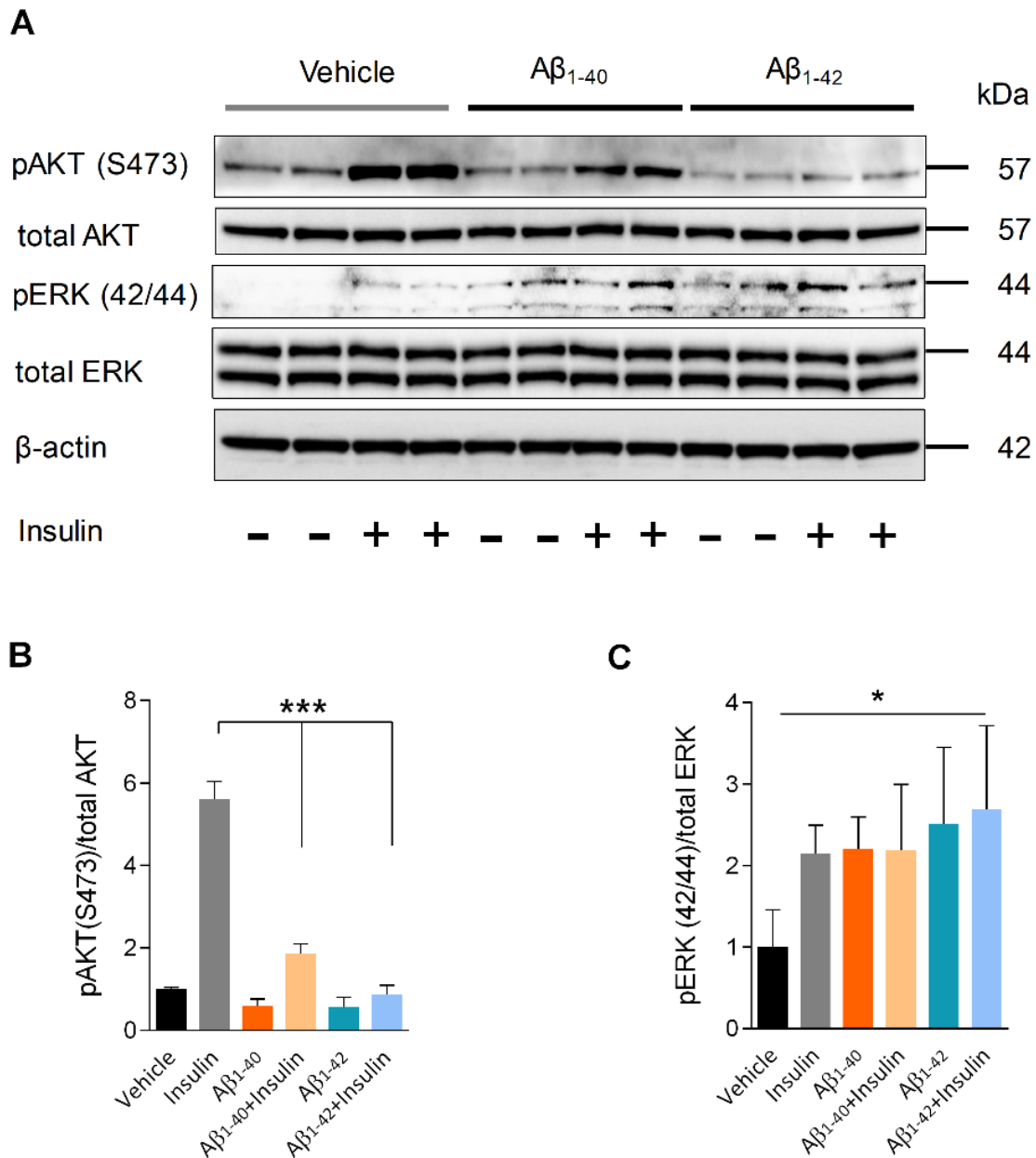


Figure 29: Effects of A β_{1-40} or A β_{1-42} peptides on insulin signaling in pBCEC.

Cells were treated with vehicle (0.01N HCL and 1% NH₄OH), insulin (100 nM), A β_{1-40} or A β_{1-42} peptides (240 nM), or a combination of insulin and A β_{1-40} or A β_{1-42} for 30 min. Cell lysates were prepared and subjected to WB using specific primary antibodies. (A) Representative western blots for pAKT, total AKT, pERK, total ERK and β -actin. Quantitative analysis of (B) pAKT and (C) pERK, bands were normalized to their respective total AKT and ERK intensities; n=4 experiments from different cell preparations. Data represent mean $\pm$ SD. Statistically significant differences were calculated using one-way ANOVA, followed by Dunnett's post-hoc test. *p<0.05, **p<0.01, and ***p<0.001. [Reproduced from Gali CC., *et al*, (Fig. 7D-F) with the permission from Mol Cell Neurosci (1)]

4.2.4 **A β treatment accelerates LRP-1 and IR- β degradation via autophagy-lysosomal induction in pBCEC**

Our findings in 3XTg-AD mice depicted a correlation between elevated cerebral and cerebrovascular LC3B levels (**Fig. 26C-D**), cerebral A β burden (**Fig. 19A&B**), and reduced LRP-1 and IR- β receptors in brain and cerebromicrovasculature (**Fig. 24A-C**). Double IF stainings revealed that A β_{1-40} peptide incubation enhanced LAMP-1 levels and reduced LRP-1 and IR- β levels in pBCEC (**Fig. 30A&D**). Based on the above findings, we hypothesized that A β treatment may drive LRP-1 and IR- β receptors towards the autophagy-lysosomal pathways and enhance their degradation. To understand this phenomenon, under SF conditions, pBCEC were incubated with A β_{1-40} peptide (predominant soluble A β peptide in CAA pathology) for 6 h, with or without autophagy-lysosomal inhibitor (chloroquine; 30 μ M) and proteasome inhibitor (MG132; 20 μ M). Interestingly, both chloroquine and MG132 treatment rescued A β_{1-40} peptide mediated LRP-1 and IR- β levels (**Fig. 30B&D**). Chloroquine significantly inhibited A β_{1-40} induced lysosomal induction, as depicted by significant reduction in LAMP-1 levels, an increase in LC3B accumulation, and a significant reduction in autophagy genes ATG 5 and ATG 3, as well as in proteasomal markers 26S proteasome non-ATPase regulatory subunit 4 (PSMD4) and heat shock protein 27 (HSP27) (**Fig. 30B-D**). Chloroquine treatment attributed to maximal recovery of A β derived LRP-1 and IR- β degradation. Even though MG132 reduced A β_{1-40} peptide induced LAMP-1 levels in pBCEC, it partially rescued LRP-1 and IR- β levels (**Fig. 30B&D**). These findings suggest that A β burden/trafficking in pBCEC accelerates LRP-1 and IR- β degradation.

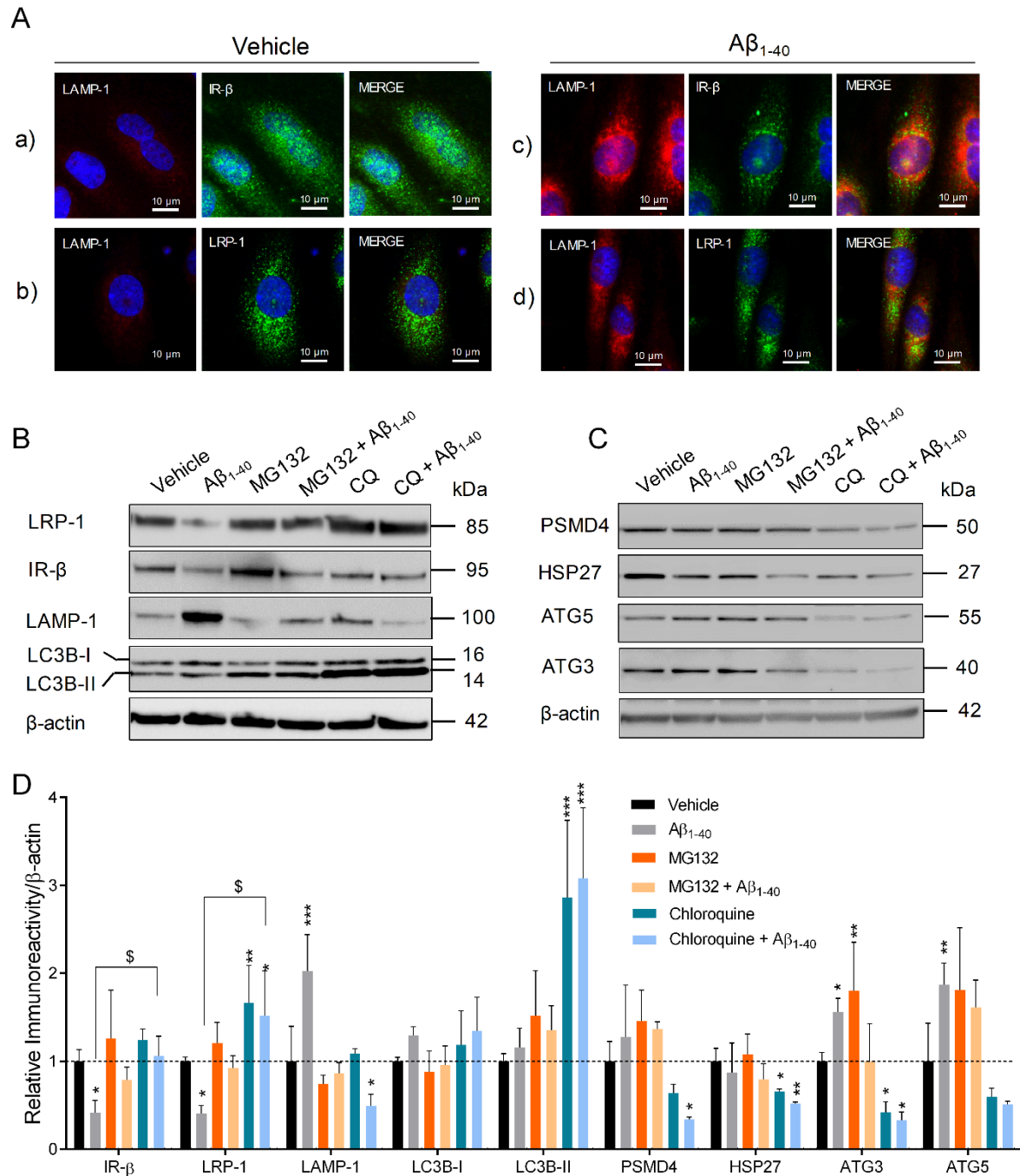


Figure 30: Effects of $A\beta_{1-40}$ treatment on LRP-1/ IR- β levels and LAMP-1 in pBCEC.

Primary pBCEC were seeded and grown on SuperFrost chamber slides. Cells were serum starved for 6 h and incubated with vehicle or freshly reconstituted $A\beta_{1-40}$ (240 nM) for 6 h. (A) Double IF stainings of (a, c) LAMP-1 (red) and IR- β (green), (b, d) LAMP-1 (red) and LRP-1 (green). pBCEC were grown on 6-well plates. Cells were incubated with vehicle or $A\beta_{1-40}$ (240 nM), with or without chloroquine (30 μ M) and MG132 (20 μ M) for 6 h under SF conditions, cells were lysed and subjected to WB (B, C) Representative western blots for LRP-1, IR- β , LAMP-1, LC3B, PSMD4, HSP27, ATG5, and ATG3 protein expression. (D) Densitometric evaluation of the proteins listed in B, C. Band intensities were normalized to β -actin. Data represent mean $\pm$ SD. Statistically significant differences were calculated using one-way ANOVA, followed by Dunnett's post-hoc test (* p <0.05, ** p <0.01, and

*** $p < 0.001$) and unpaired students t-test ($^*p < 0.05$). [Reproduced from Gali CC., *et al*, (Fig. 8) with the permission from Mol Cell Neurosci (1)]

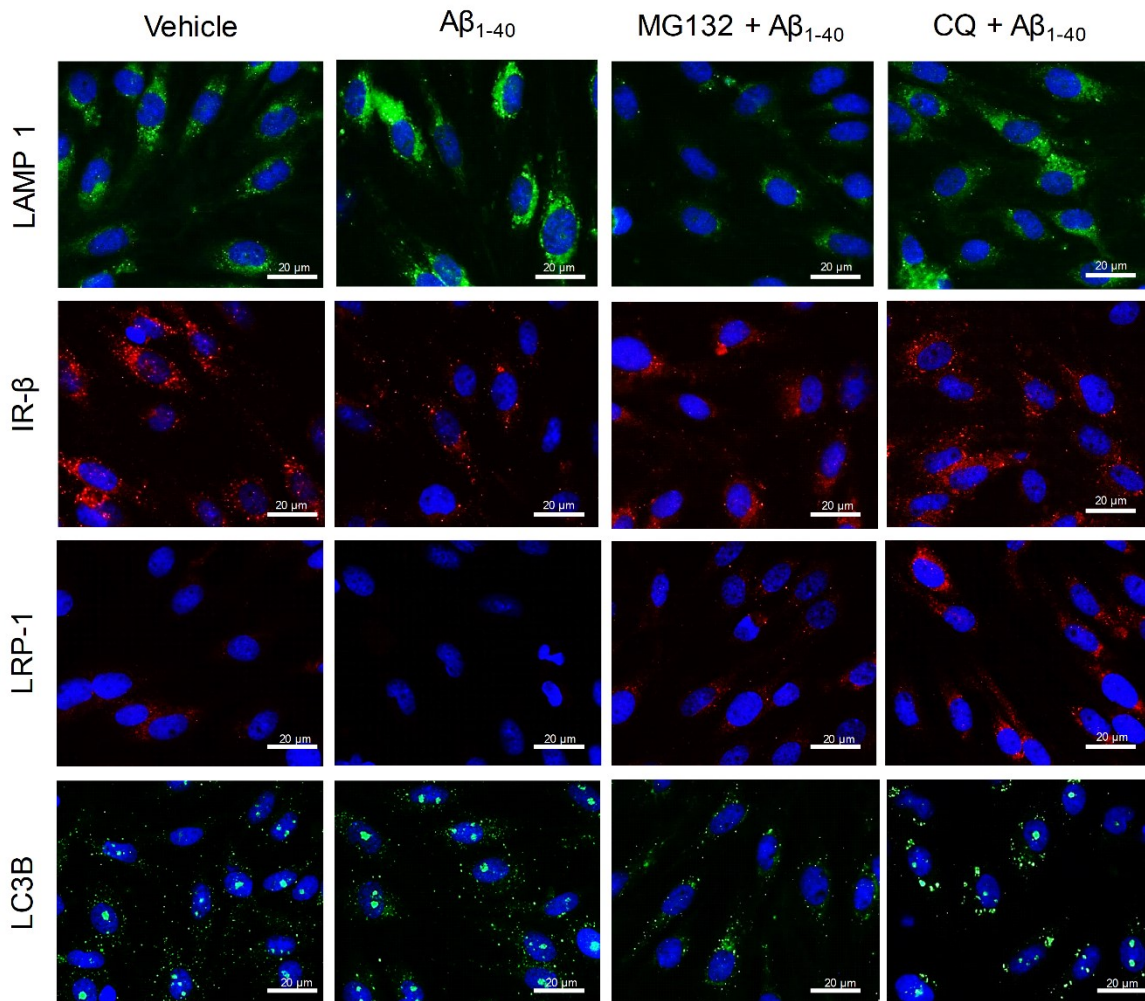


Figure 31: Immunofluorescent staining depicting the effect of lysosomal and proteasomal inhibitors on $A\beta_{1-40}$ induced LRP-1 and IR- β impairment and LAMP-1 induction in pBCEC.

Cells were grown on SuperFrost chamber slides, serum starved for 6 h and treated with $A\beta_{1-40}$ peptide with or without chloroquine (30 μ M) and MG132 (20 μ M) for 6 h, 4% PFA fixed and IF stained with LAMP-1 (green; a-d), IR- β (red; e-h), LRP-1 (red; i-l) and LC3B (green; m-p). Nuclear staining (DAPI) in blue. [Reproduced from Gali CC., *et al*, (Fig. 8E) with the permission from Mol Cell Neurosci (1)]

4.2.5 LRP-1 silencing disrupts IR- β levels and insulin signaling in pBCEC

Given the effects of $A\beta$ on IR- β and LRP-1 expression and their interaction in pBCEC (**Fig. 28**), we next assessed a possible crosstalk between LRP-1 and IR- β that might engender these effects. To address this, pBCEC were transfected with either LRP-1, IR- β , or mock (non-targeting control, NTC) silencing (si)RNAs (materials and methods section 1.5). Gene silencing efficiency was confirmed by mRNA

expression levels of LRP-1 and IR- β using RT-qPCR (**Fig. 32A&B**). LRP-1 silencing significantly reduced IR- β protein levels in pBCEC (**Fig. 32C-E**). Moreover, LRP-1 silencing impaired insulin mediated activation of IR- β and downstream signaling, depicted by significantly low activation of IR- β (Tyr1131) and AKT (S473) in LRP-1 silenced pBCEC (**Fig. 32C&D**). Interestingly, IR- β silencing had no effect on LRP-1 levels but significantly reduced upstream (IR) and downstream (AKT) insulin signaling (**Fig. 32C-G**). In summary, these results suggest that LRP-1 is associated with IR- β receptor expression and is important for insulin signaling in BBB endothelial cells and loss in LRP-1 may lead to impairment in IR- β levels, which in turn may effect insulin signaling.

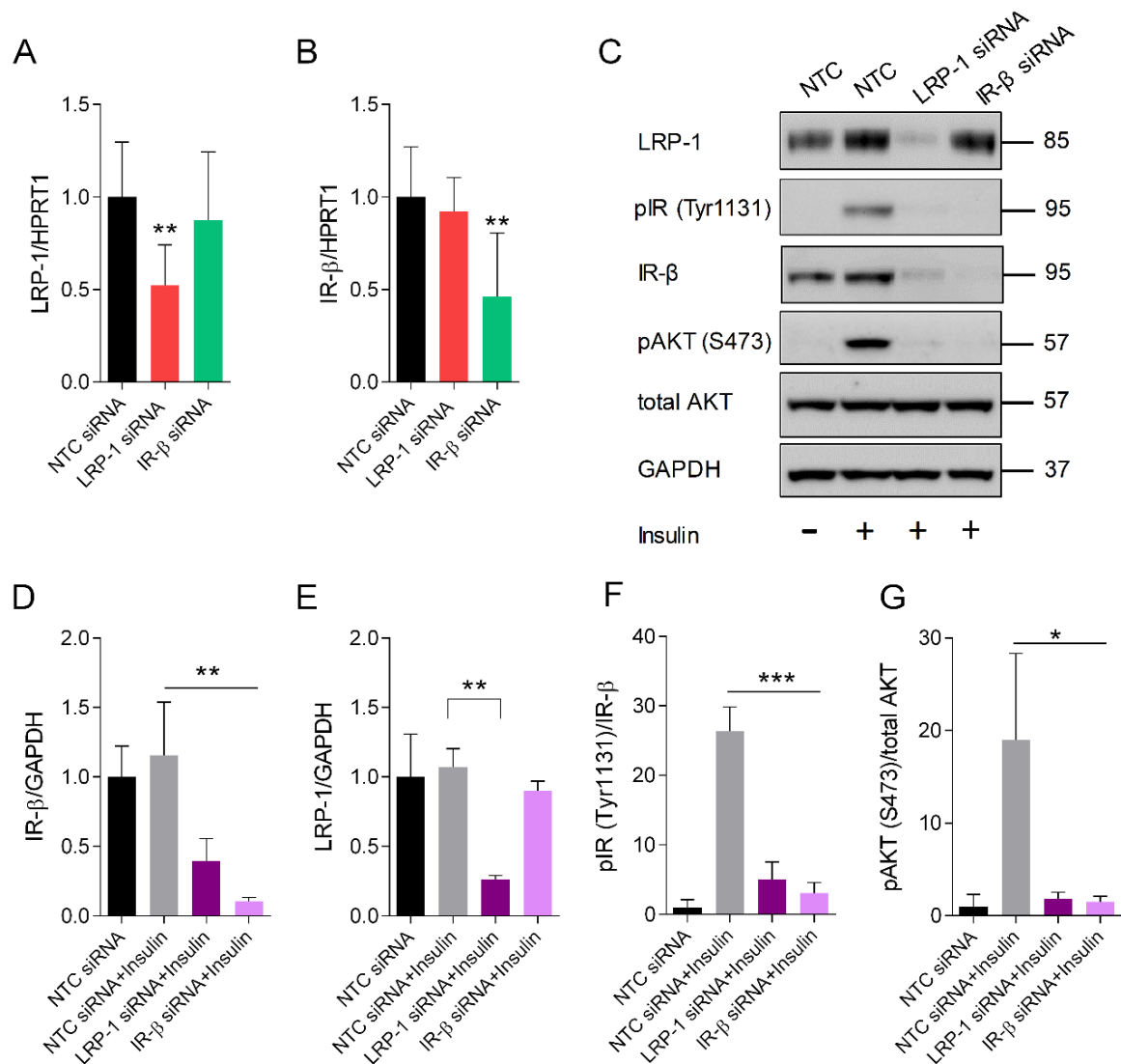


Figure 32: Role of LRP-1 in BBB endothelial IR- β regulation and insulin signaling.

Specific gene silencing in pBCEC was achieved by RNA interference using either NTC siRNA, LRP-1 siRNA, or IR- β siRNA for 48 h. Gene silencing efficiency was confirmed by subjecting isolated

cDNA to RT-qPCR. (A) LRP-1 mRNA levels and (B) IR- β mRNA levels, normalized to HPRT1. n=3 experiments, statistically significant differences were calculated using one-way ANOVA, followed by Dunnett's post-hoc test; *p<0.05; **p<0.01 and ***p<0.001. Silenced cells were serum starved for 12 h and treated with either vehicle (0.01N HCL) or insulin (100 nM solubilized in 0.01N HCL) for 5 min to induce pIR (Tyr1131) and pAKT (S473). After incubation, cells were lysed, and proteins were subjected to IB using respective antibodies. (C) Representative western blots for LRP-1, pIR (Tyr1131), IR- β , pAKT (S473), total AKT and GAPDH (D) Quantitative analysis of (D) IR- β and (E) LRP-1, where the band intensities were normalized to GAPDH; Quantitative analysis of (F) pAKT (S473) and (G) pIR (Tyr1131), where the band intensities were normalized to respective total protein band intensities. Data represents mean $\pm$ SD. n=3 experiments, statistically significant differences were calculated using one-way ANOVA, followed by Dunnett's post-hoc test; *p<0.05; **p<0.01 and ***p<0.001. [Reproduced from Gali CC., *et al*, (Fig. 9) with the permission from Mol Cell Neurosci (1)]

5. DISCUSSION

[Parts of this chapter have been literally published in “Gali CC, Fanaee-Danesh E, Zandi-Lang M, Albrecher NM, Tam-Amersdorfer C, Stracke A, et al. Amyloid-beta impairs insulin signaling by accelerating autophagy-lysosomal degradation of LRP-1 and IR-beta in blood-brain barrier endothelial cells in vitro and in 3XTg-AD mice. *Mol Cell Neurosci*. 2019 Jul 2;99:103390.”, with the permission of reuse from *Mol Cell Neurosci*]

A growing body of evidence associates AD with characteristics of diabetes, and impaired brain insulin signaling is known to be one of the early hallmarks of AD (121, 124, 143, 215). Various histopathological findings in human AD brain samples identified changes in insulin signaling pathways and receptors involved in insulin signaling (117, 120). Similarly, early changes in brain insulin signaling and their machinery have been described previously in different AD mouse models (195, 216). Recently, anti-diabetic drugs and insulin therapies gained popularity and have been tested successfully in AD mouse models and undergone several clinical trials to establish their therapeutic potential against memory related disorders like AD, mild cognitive impairment (MCI), and other types of dementia (72, 140, 217-219). Even though anti-diabetic therapies promise to have therapeutic potential in AD, the factors and molecular mechanisms causing the changes in cerebral and cerebrovascular endothelial insulin signaling still remain elusive, restricting to achieve a targeted therapy. Herein, using an AD transgenic mouse model and porcine *in vitro* BBB model, we aimed to uncover the key factors and molecular mechanisms modulating cerebrovascular endothelial insulin signaling in AD.

We implemented a HFD feeding protocol to understand the impact of western diet feeding on central and peripheral insulin signaling and brain A β burden. Previously, it has been demonstrated that HFD feeding in 3XTg-AD mice accelerates AD progression by hindering peripheral insulin release, leading to decrease in brain A β clearance and increase in brain A β burden, in turn dampening memory in mice (108, 207). Interestingly, it has been shown that a single dose of insulin improved memory by accelerating A β clearance from brain in 3XTg-AD mouse model (207). Moreover, A β was found to accumulate in pancreatic islets where insulin is produced, leading to apoptosis of pancreatic cells, thereby hindering the release of insulin and causing

glucose intolerance (206, 207). Gender distinctions related to feeding habits (220), weight gain (221), severity of AD (222), and A β accumulation (223) were reported. In this thesis, keeping this mind, we employed both male and female 3XTg-AD and NTg mice to delineate the gender distinctions on the outcome of the study.

During the course of the feeding study body weight gain and food intake were monitored, distinctions in food intake (**Fig. 17C**) and body weight gain (**Fig. 17A**) were obvious between both sexes and genotypes. HFD feeding in male and female mice led to an increase in body weight gain (**Fig. 17A**), plasma cholesterol (**Fig. 18D**), and glucose intolerance (**Fig. 17C**). Interestingly, lower food intake was observed in HFD fed NTg female mice when compared to female 3XTg-AD mice (**Fig. 17C**), as reflected by lower plasma cholesterol levels and body weight gain, but developed glucose intolerance similar to female 3XTg-AD mice (**Fig. 17B**). Genotype, sex, and diet had a significant impact on fasting plasma insulin levels in mice (**Fig. 18A**). Gender distinctions in plasma insulin levels were reported by others (221, 224). Similarly, in this thesis, male mice showed higher fasting plasma insulin levels, when compared to females in both genotypes (**Fig. 18A**). HFD feeding elevated fasting plasma insulin levels in both the sexes and transgenes, corresponding with glucose intolerance, and elevated BW gain. In line with earlier findings by Vandal et al., 2014 (207), HFD feeding in 3XTg-AD mice did not elevate plasma insulin levels as opposed to NTg mice, strengthening the observation that HFD feeding in 3XTg-AD mice tends to enhance pancreatic islet A β deposits (206, 207), in turn leading to decreased insulin release from pancreatic islets. Elevated plasma NEFA (**Fig. 18B**) and triglyceride levels (**Fig. 18C**) in 3XTg-AD mice and elevated plasma cholesterol levels (**Fig. 18D**) in 3XTg-AD female mice indicate altered metabolic profile due to the transgenes and/or A β pathology. The increase in plasma triglyceride levels in 3XTg-AD mice supports the earlier findings by Nagga et al., 2018 and Burgess et al., 2006, where elevated plasma triglyceride levels were reported to precede A β deposition in Tg-CRND8 AD mouse model and abnormal CSF A β ₁₋₄₂ levels in cognitively normal individuals (225, 226). Gender distinctions and hormonal profile can have significant effects on plasma lipid parameters across genotypes (227).

The liver is the major peripheral organ which regulates degradation and clearance of complex molecules and numerous metabolites produced in the body. Recent evidence supports that hepatocytes regulate peripheral A β clearance via LRP-1 mediated uptake and degradation (208, 209). Moreover, studies in mice showed that insulin treatment can improve the surface expression of LRP-1 and enhance the rate of A β clearance by hepatocytes (209). In mice, hepatic LRP-1 was shown to regulate insulin responses and energy homeostasis via leptin signaling; its deletion caused peripheral or hepatic insulin resistance thereby causing enhanced body weight gain, dyslipidemia, glucose intolerance, and low surface IR expression (228, 229). HFD feeding in both genotypes and sexes caused impairment in insulin sensitivity (reduced hepatic pAKT levels) and reduced liver LRP-1 levels (**Fig. 21B&C**). More importantly, reduced LRP-1 levels in HFD fed 3XTg-AD mice correlated with elevated brain A β burden in brain (**Fig. 19A-C**). Functional hepatic LRP-1 is important for peripheral (liver) A β degradation and/or clearance, 3XTg-AD mice demonstrated impaired IR- β levels (**Fig. 21C**), which may hinder insulin mediated hepatic A β uptake via LRP-1 and thereby may hamper peripheral A β degradation.

Amyloid- β accumulation in brain, primarily hippocampus and cortex, is associated with synaptic loss and is known to impair memory in mice and humans (230-232). Insulin therapeutics are known to enhance rapid clearance of A β from brain and periphery, partly via LRP-1 mediated pathways (206, 233). Similarly, insulin stimulation is known to enhance enzymatic degradation of A β products produced in brain by stimulating IDE release or production, aiding faster A β degradation (234, 235). Under insulin deficit/resistant conditions, A β peptides accumulate in brain, since insulin is non-reactive towards its own receptors (IRs) and functional regulation of LRP-1. In line with the previous reports (222, 223, 236), we identified that female 3XTg-AD mice develop aggressive AD pathology compared to male mice, as understood by very high levels of soluble and insoluble A β levels in female 3XTg-AD mice (**Fig. 19A-C**). Similarly, female 3XTg-AD mice performed worse in memory assessment (BM) and showed elevated levels of anxiety (**Fig. 20A-D**), which was gender specific and correlated with higher levels of A β accumulation when compared with male 3XTg-AD mice. HFD feeding worsened memory in female 3XTg-AD mice (**Fig. 20A-D**), but had no effect on male mice, which correlates with elevated brain A β levels in female mice.

Insulin signaling, mediated by IR is key and was reported to be involved in hippocampal synaptic plasticity and memory (127, 237-239). Insulin signaling is key for GLUT 4 mediated glucose uptake in neuronal cells (159). Impairment in insulin signaling leads to insulin resistance, predominantly due to abolished PI3K signaling, which is associated with cognitive performance (125, 240). Subsequently, it was understood that LRP-1 is critical for brain insulin signaling, and its deletion in mouse brain can lead to impaired insulin receptor levels and dampen insulin signaling, leading to reduced glucose uptake in neurons by altering the levels of GLUT 3 and 4 transporters (159, 161, 229). Similar to findings reported in neuronal LRP-1 KO mice (159), in 3XTg-AD mouse brain lysates we identified impaired LRP-1 levels and insulin signaling (**Fig. 22**), denoted by reduced IR- β (**Fig. 22D**) and pIRS-1(S1101) (**Fig. 22E**) levels, and hyperactive downstream insulin signaling, indicated by elevated pAKT (**Fig. 20F**) and pERK levels (**Fig. 22G**). HFD feeding did not show significant changes in brain IR- β levels or insulin signaling in 3XTg-AD mice, limiting its potential to alter predominantly downstream insulin signaling, indicated by reduced pAKT levels (**Fig. 22F**) and elevated pERK levels (**Fig. 22G**) in brain. Hyperactivation of downstream insulin signaling was noticed in 3XTg-AD mice brain (indicated by elevated pAKT levels), which is known to associate with “selective insulin resistance”, (195, 241), a paradoxical downstream insulin signaling triggering state, which may disrupt upstream insulin signaling via downregulating phosphorylation events of upstream mediators of IR and receptor associated proteins. Even though ERK is induced in response to insulin, known to positively modulate cell growth, it can also disrupt insulin signaling via TNF α -induced insulin resistance (242-244).

Cerebrovascular endothelium is the rate limiting barrier for metabolites trafficking from brain to blood and vice versa (245). As outlined in more detail in the introduction, A β produced in brain is known to traffic across BBB using various receptors mainly apoE family receptors, more importantly LRP-1 and LRP-2 (212, 246). LRP-1 works in tandem with CD31 and Pgp to traffic A β across the BBB (211). Interestingly, insulin was shown to enhance surface expression of LRP-1 in a human endothelial cell line (233), and may modulate A β uptake and trafficking at the cerebrovascular endothelium. Recently, it has been identified that cerebrovascular LRP-1 levels are downregulated in aged 3XTg-AD mice (247). In this thesis, we confirmed that mouse cerebromicrovascular endothelium expresses LRP-1 and IR- β

(**Fig. 23B**), and their protein levels are significantly impaired in 9-month-old male and female 3XTg-AD mice (**Fig. 23A-C**), further strengthening our argument that enhanced cerebral A β burden correlates with impaired LRP-1 and IR- β levels in cerebrovascular endothelium of 3XTg-AD mice.

We and others have showed that 3XTg-AD mice develop cerebral/neuronal A β deposition very early (200), as confirmed in the present thesis, given by brain soluble and insoluble brain A β levels (**Fig. 19A&B**), APP/A β (6E10) IF staining (**Fig. 19C**), and Congo red staining (A β plaques) in male and female mice (**Fig. 25A&B**). Occurrence of CAA, primarily represented by cerebrovascular accumulation of insoluble A β fibrils was not apparent in 9-month-old 3XTg-AD female or male mice, used in our study (**Fig. 24C-H**). Moreover, the cerebrovascular endothelium is known to uptake and transport soluble A β , predominantly through endothelial LRP-1, which is known to regulate about 50% of total cerebral A β clearance in a forced AD (5XFAD) mouse model (212). It is likely that elevated A β burden in brain can interact with the BBB under conditions of early AD, as seen in 3XTg-AD mice.

In pBCEC, A β treatment lead to impairment in LRP-1 and IR- β receptors (**Fig. 28A**) and their interaction (**Fig. 28C**), in association with impairment in insulin mediated signaling (**Fig. 29**), which supports the finding that A β competes with insulin for IR. In pBCEC, insulin treatment elevated the levels of LRP-1 (**Fig. 28A&B**) and maintained IR- β levels, probably by diverting LRP-1 and IR- β vesicles away from lysosomal or proteasome degradation. Autophagy-lysosomal and proteasomal degradation are key processes regulating degradation of cell proteins and are important in maintaining proteostasis (248, 249). Both have the capacity to degrade many fully folded as well as misfolded and aggregated proteins to alleviate the protein stress. Since A β peptides are amyloidogenic, known to aggregate and possess oligomeric properties, and can induce proteostasis-induced endoplasmic reticulum stress (250), protein degradation pathways play a primordial role in degrading these misfolded proteins. Similarly, surface receptors and proteins are recycled through autophagy-lysosomal and proteasomal degradation pathways (251, 252). Amyloid β has propensity to bind with both the LRP-1 and IR- β receptors, and may divert these receptors towards lysosomal and/or proteasomal degradation. Our findings in pBCEC revealed that A β_{1-40} treatment strongly induced lysosomal activity, which is indicated by elevated LAMP-1 levels (**Fig. 30A&B**) and ATG's (**Fig. 30D**) involved

in autophagosome formation, treatment with lysosomal inhibitor (chloroquine) or proteasomal inhibitor (MG132) rescued A β mediated LRP-1 and IR- β degradation in pBCEC (**Fig. 30B&D**). Our observations were strengthened by the fact that 3XTg-AD mice showed elevated cerebral and cerebrovascular LC3B levels (**Fig. 26**), indicating elevated autophagy. Moreover, HFD feeding in 3XTg-AD (M/F) mice enhanced cerebromicrovascular LC3B levels, which correlates with elevated cerebral A β burden (**Fig. 24**). Chloroquine showed prominent effects on A β induced LRP-1 and IR- β degradation when compared with MG132 treatment (**Fig. 30B-D**), indicating that A β diverts these receptors, predominantly towards lysosomal degradation, which may contribute to disrupted insulin signaling responses in cerebrovascular endothelium.

Liu *et al* established that LRP-1 is critical for IR- β expression and its deletion adversely affects insulin signaling in neuronal cells (159, 161). To understand the relationship between LRP-1 and IR- β at the BBB level, we silenced both LRP-1 and IR- β gene expression in pBCEC using siRNAs targeting these receptors. LRP-1 silencing in pBCEC was associated with reduction in IR- β protein levels and insulin mediated signaling in pBCEC (**Fig. 32C-E**), whereas, silencing of IR- β had no impact on LRP-1 protein levels or expression but inhibited insulin mediated signaling (**Fig. 32C**). The mechanisms by which LRP-1 silencing alters post-translational modification of IR- β was of further investigation and remains elusive. Together, our findings indicate that altered LRP-1 levels can greatly influence insulin signaling at cerebrovascular endothelium and can blunt insulin-mediated effects (**Fig. 32C-G**).

In summary, we demonstrated impaired central and peripheral insulin signaling in 3XTg-AD mouse model, accompanied with changes in cerebral LRP-1 and IR- β levels, which correlated with altered plasma insulin levels and lipid profile. More importantly, cerebrovascular LRP-1 and IR- β levels were impaired in 3XTg-AD mice, which are crucial for A β trafficking and insulin signaling. HFD feeding elevated cerebral A β burden, plasma cholesterol levels and glucose intolerance in 3XTg-AD mice and contributed to memory impairment in female 3XTg-AD mice. Using the porcine *in vitro* BBB model, we identified that alterations in LRP-1 and IR- β were caused by A β burden, and were coupled with impaired BBB insulin signaling. Mechanistic studies revealed that A β accelerates LRP-1 and IR- β degradation, predominantly via autophagy-lysosomal induction. We identified that LRP-1 is important for

insulin signaling at the BBB, and loss in LRP-1 expression can adversely affect IR- β levels, thereby altering insulin mediated responses. Amyloid- β derived pathways regulating LRP-1 and IR- β dysregulation at the BBB are summarized in **Fig. 33**.

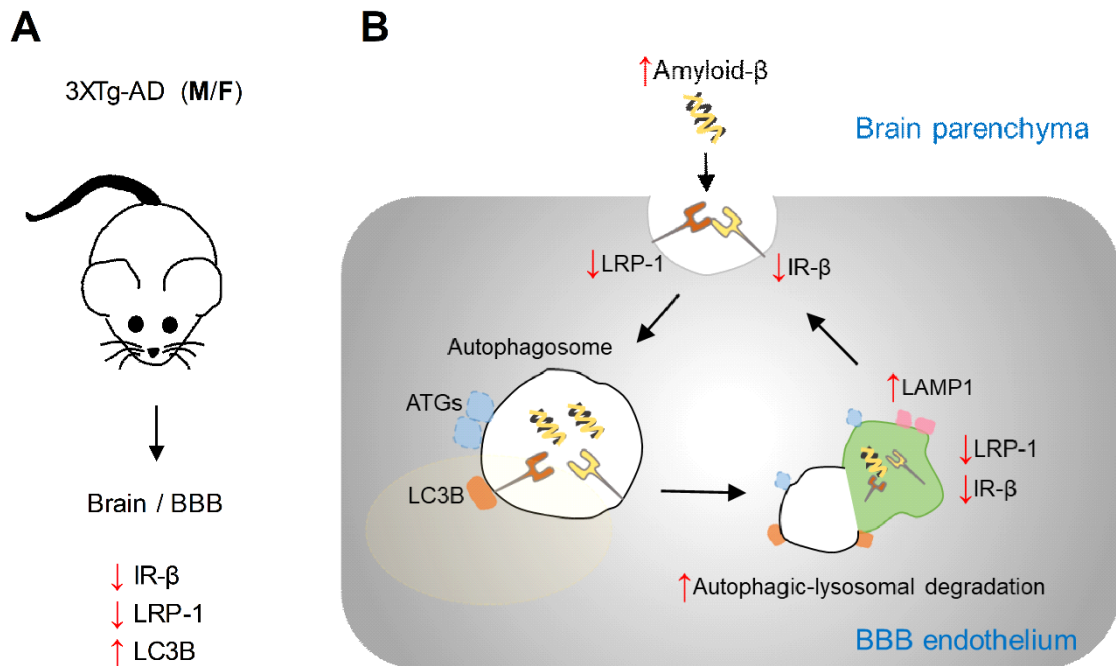


Figure 33: Diagrammatic representation of processes involved in A β mediated degradation of receptors important for insulin signaling at the BBB.

A) Impaired IR- β and LRP-1 receptor levels and elevated autophagy (LC3B) was observed in the brain and cerebrovasculature of male and female 3XTg-AD mice. B) Amyloid- β burden (in 3XTg-AD mice) or challenge (in BBB endothelium) induce autophagy-lysosomal pathways, represented by elevated ATGs, LC3B and LAMP-1 levels, in turn accelerating LRP-1 and IR- β trafficking to lysosomes for degradation. LRP-1 interacts with IR- β and mediates insulin responses in BBB endothelium, and impairment in LRP-1 and IR- β receptors hampers insulin mediated responses at the BBB. [Reproduced from Gali CC., *et al*, (Abstract Fig) with the permission from Mol Cell Neurosci (1)]

6. BIBLIOGRAPHY

1. Gali CC, Fanaee-Danesh E, Zandl-Lang M, Albrecher NM, Tam-Amersdorfer C, Stracke A, et al. Amyloid-beta impairs insulin signaling by accelerating autophagy-lysosomal degradation of LRP-1 and IR-beta in blood-brain barrier endothelial cells in vitro and in 3XTg-AD mice. *Mol Cell Neurosci*. 2019;103390.
2. Schachter AS, Davis KL. Alzheimer's disease. *Dialogues Clin Neurosci*. 2000;2(2):91-100.
3. Masters CL, Bateman R, Blennow K, Rowe CC, Sperling RA, Cummings JL. Alzheimer's disease. *Nat Rev Dis Primers*. 2015;1:15056.
4. Bird TD. Alzheimer Disease Overview. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, et al., editors. *GeneReviews*((R)). Seattle (WA)1993.
5. Todd S, Barr S, Passmore AP. Cause of death in Alzheimer's disease: a cohort study. *QJM*. 2013;106(8):747-53.
6. Alzheimer's A. 2016 Alzheimer's disease facts and figures. *Alzheimers Dement*. 2016;12(4):459-509.
7. O'Brien RJ, Wong PC. Amyloid precursor protein processing and Alzheimer's disease. *Annu Rev Neurosci*. 2011;34:185-204.
8. Bayer TA, Cappai R, Masters CL, Beyreuther K, Multhaup G. It all sticks together--the APP-related family of proteins and Alzheimer's disease. *Mol Psychiatry*. 1999;4(6):524-8.
9. Huse JT, Liu K, Pijak DS, Carlin D, Lee VM, Doms RW. Beta-secretase processing in the trans-Golgi network preferentially generates truncated amyloid species that accumulate in Alzheimer's disease brain. *J Biol Chem*. 2002;277(18):16278-84.
10. Greenfield JP, Tsai J, Gouras GK, Hai B, Thinakaran G, Checler F, et al. Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer beta-amyloid peptides. *Proc Natl Acad Sci U S A*. 1999;96(2):742-7.
11. Wang Z, Xu Q, Cai F, Liu X, Wu Y, Song W. BACE2, a conditional beta-secretase, contributes to Alzheimer's disease pathogenesis. *JCI Insight*. 2019;4(1).
12. Wang N, Chen J, Xiao H, Wu L, Jiang H, Zhou Y. Application of artificial neural network model in diagnosis of Alzheimer's disease. *BMC Neurol*. 2019;19(1):154.

13. Eisenberg DS, Sawaya MR. Implications for Alzheimer's disease of an atomic resolution structure of amyloid-beta(1-42) fibrils. *Proc Natl Acad Sci U S A*. 2016;113(34):9398-400.
14. Wang J, Gu BJ, Masters CL, Wang YJ. A systemic view of Alzheimer disease - insights from amyloid-beta metabolism beyond the brain. *Nat Rev Neurol*. 2017;13(10):612-23.
15. Brandon JA, Farmer BC, Williams HC, Johnson LA. APOE and Alzheimer's Disease: Neuroimaging of Metabolic and Cerebrovascular Dysfunction. *Front Aging Neurosci*. 2018;10:180.
16. Metaxas A, Kempf SJ. Neurofibrillary tangles in Alzheimer's disease: elucidation of the molecular mechanism by immunohistochemistry and tau protein phospho-proteomics. *Neural Regen Res*. 2016;11(10):1579-81.
17. Stancu IC, Vasconcelos B, Terwel D, Dewachter I. Models of beta-amyloid induced Tau-pathology: the long and "folded" road to understand the mechanism. *Mol Neurodegener*. 2014;9:51.
18. Singh SK, Srivastav S, Yadav AK, Srikrishna S, Perry G. Overview of Alzheimer's Disease and Some Therapeutic Approaches Targeting Abeta by Using Several Synthetic and Herbal Compounds. *Oxid Med Cell Longev*. 2016;2016:7361613.
19. Selkoe DJ. Clearing the brain's amyloid cobwebs. *Neuron*. 2001;32(2):177-80.
20. Tarasoff-Conway JM, Carare RO, Osorio RS, Glodzik L, Butler T, Fieremans E, et al. Clearance systems in the brain-implications for Alzheimer disease. *Nat Rev Neurol*. 2015;11(8):457-70.
21. Yao XQ, Jiao SS, Saadipour K, Zeng F, Wang QH, Zhu C, et al. p75NTR ectodomain is a physiological neuroprotective molecule against amyloid-beta toxicity in the brain of Alzheimer's disease. *Mol Psychiatry*. 2015;20(11):1301-10.
22. Liao MC, Hoos MD, Aucoin D, Ahmed M, Davis J, Smith SO, et al. N-terminal domain of myelin basic protein inhibits amyloid beta-protein fibril assembly. *J Biol Chem*. 2010;285(46):35590-8.
23. Qosa H, Abuasal BS, Romero IA, Weksler B, Couraud PO, Keller JN, et al. Differences in amyloid-beta clearance across mouse and human blood-brain barrier models: kinetic analysis and mechanistic modeling. *Neuropharmacology*. 2014;79:668-78.

24. Yuede CM, Lee H, Restivo JL, Davis TA, Hettinger JC, Wallace CE, et al. Rapid in vivo measurement of beta-amyloid reveals biphasic clearance kinetics in an Alzheimer's mouse model. *J Exp Med*. 2016;213(5):677-85.
25. Bradshaw EM, Chibnik LB, Keenan BT, Ottoboni L, Raj T, Tang A, et al. CD33 Alzheimer's disease locus: altered monocyte function and amyloid biology. *Nat Neurosci*. 2013;16(7):848-50.
26. Kanekiyo T, Bu G. The low-density lipoprotein receptor-related protein 1 and amyloid-beta clearance in Alzheimer's disease. *Front Aging Neurosci*. 2014;6:93.
27. Ghiso J, Calero M, Matsubara E, Governale S, Chuba J, Beavis R, et al. Alzheimer's soluble amyloid beta is a normal component of human urine. *FEBS Lett*. 1997;408(1):105-8.
28. Ghiso J, Shayo M, Calero M, Ng D, Tomidokoro Y, Gandy S, et al. Systemic catabolism of Alzheimer's Abeta40 and Abeta42. *J Biol Chem*. 2004;279(44):45897-908.
29. Liu Z, Zhu H, Fang GG, Walsh K, Mwamburi M, Wolozin B, et al. Characterization of insulin degrading enzyme and other amyloid-beta degrading proteases in human serum: a role in Alzheimer's disease? *J Alzheimers Dis*. 2012;29(2):329-40.
30. Biere AL, Ostaszewski B, Stimson ER, Hyman BT, Maggio JE, Selkoe DJ. Amyloid beta-peptide is transported on lipoproteins and albumin in human plasma. *J Biol Chem*. 1996;271(51):32916-22.
31. Kuo YM, Kokjohn TA, Kalback W, Luehrs D, Galasko DR, Chevallier N, et al. Amyloid-beta peptides interact with plasma proteins and erythrocytes: implications for their quantitation in plasma. *Biochem Biophys Res Commun*. 2000;268(3):750-6.
32. Weiss M, Steiner DF, Philipson LH. Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. *Endotext*. South Dartmouth (MA)2000.
33. Fu Z, Gilbert ER, Liu D. Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. *Curr Diabetes Rev*. 2013;9(1):25-53.
34. Komatsu M, Takei M, Ishii H, Sato Y. Glucose-stimulated insulin secretion: A newer perspective. *J Diabetes Investig*. 2013;4(6):511-6.

35. Wilcox G. Insulin and insulin resistance. *Clin Biochem Rev.* 2005;26(2):19-39.
36. Yang Y, Hua QX, Liu J, Shimizu EH, Choquette MH, Mackin RB, et al. Solution structure of proinsulin: connecting domain flexibility and prohormone processing. *J Biol Chem.* 2010;285(11):7847-51.
37. Watson GS, Craft S. The role of insulin resistance in the pathogenesis of Alzheimer's disease: implications for treatment. *CNS Drugs.* 2003;17(1):27-45.
38. Banks WA, Jaspan JB, Huang W, Kastin AJ. Transport of insulin across the blood-brain barrier: saturability at euglycemic doses of insulin. *Peptides.* 1997;18(9):1423-9.
39. Banks WA, Jaspan JB, Kastin AJ. Selective, physiological transport of insulin across the blood-brain barrier: novel demonstration by species-specific radioimmunoassays. *Peptides.* 1997;18(8):1257-62.
40. Baskin DG, Figlewicz DP, Woods SC, Porte D, Jr., Dorsa DM. Insulin in the brain. *Annu Rev Physiol.* 1987;49:335-47.
41. Baura GD, Foster DM, Porte D, Jr., Kahn SE, Bergman RN, Cobelli C, et al. Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. *J Clin Invest.* 1993;92(4):1824-30.
42. Gray SM, Aylor KW, Barrett EJ. Unravelling the regulation of insulin transport across the brain endothelial cell. *Diabetologia.* 2017;60(8):1512-21.
43. Yazdani S, Jaldin-Fincati JR, Pereira RVS, Klip A. Endothelial cell barriers: Transport of molecules between blood and tissues. *Traffic.* 2019;20(6):390-403.
44. Schulingkamp RJ, Pagano TC, Hung D, Raffa RB. Insulin receptors and insulin action in the brain: review and clinical implications. *Neurosci Biobehav Rev.* 2000;24(8):855-72.
45. Apelt J, Mehlhorn G, Schliebs R. Insulin-sensitive GLUT4 glucose transporters are colocalized with GLUT3-expressing cells and demonstrate a chemically distinct neuron-specific localization in rat brain. *J Neurosci Res.* 1999;57(5):693-705.
46. Skeberdis VA, Lan J, Zheng X, Zukin RS, Bennett MV. Insulin promotes rapid delivery of N-methyl-D- aspartate receptors to the cell surface by exocytosis. *Proc Natl Acad Sci U S A.* 2001;98(6):3561-6.

47. Rensink AA, Otte-Holler I, de Boer R, Bosch RR, ten Donkelaar HJ, de Waal RM, et al. Insulin inhibits amyloid beta-induced cell death in cultured human brain pericytes. *Neurobiol Aging*. 2004;25(1):93-103.
48. Ryu BR, Ko HW, Jou I, Noh JS, Gwag BJ. Phosphatidylinositol 3-kinase-mediated regulation of neuronal apoptosis and necrosis by insulin and IGF-I. *J Neurobiol*. 1999;39(4):536-46.
49. Kido Y, Nakae J, Accili D. Clinical review 125: The insulin receptor and its cellular targets. *J Clin Endocrinol Metab*. 2001;86(3):972-9.
50. Boucher J, Kleinridders A, Kahn CR. Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harb Perspect Biol*. 2014;6(1).
51. Poloz Y, Stambolic V. Obesity and cancer, a case for insulin signaling. *Cell Death Dis*. 2015;6:e2037.
52. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 2005;307(5712):1098-101.
53. Oh WJ, Jacinto E. mTOR complex 2 signaling and functions. *Cell Cycle*. 2011;10(14):2305-16.
54. Schultze SM, Jensen J, Hemmings BA, Tschopp O, Niessen M. Promiscuous affairs of PKB/AKT isoforms in metabolism. *Arch Physiol Biochem*. 2011;117(2):70-7.
55. Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, et al. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell*. 2010;39(2):171-83.
56. Stoeckel LE, Arvanitakis Z, Gandy S, Small D, Kahn CR, Pascual-Leone A, et al. Complex mechanisms linking neurocognitive dysfunction to insulin resistance and other metabolic dysfunction. *F1000Res*. 2016;5:353.
57. Chatterjee S, Peters SA, Woodward M, Mejia Arango S, Batty GD, Beckett N, et al. Type 2 Diabetes as a Risk Factor for Dementia in Women Compared With Men: A Pooled Analysis of 2.3 Million People Comprising More Than 100,000 Cases of Dementia. *Diabetes Care*. 2016;39(2):300-7.
58. Gao C, Liu Y, Li L, Holscher C. New animal models of Alzheimer's disease that display insulin desensitization in the brain. *Rev Neurosci*. 2013;24(6):607-15.

59. Yaffe K, Falvey C, Hamilton N, Schwartz AV, Simonsick EM, Satterfield S, et al. Diabetes, glucose control, and 9-year cognitive decline among older adults without dementia. *Arch Neurol*. 2012;69(9):1170-5.
60. Biessels GJ, Staekenborg S, Brunner E, Brayne C, Scheltens P. Risk of dementia in diabetes mellitus: a systematic review. *Lancet Neurol*. 2006;5(1):64-74.
61. Fontbonne A, Berr C, Ducimetiere P, Alperovitch A. Changes in cognitive abilities over a 4-year period are unfavorably affected in elderly diabetic subjects: results of the Epidemiology of Vascular Aging Study. *Diabetes Care*. 2001;24(2):366-70.
62. Logroscino G, Kang JH, Grodstein F. Prospective study of type 2 diabetes and cognitive decline in women aged 70-81 years. *BMJ*. 2004;328(7439):548.
63. Luchsinger JA, Reitz C, Patel B, Tang MX, Manly JJ, Mayeux R. Relation of diabetes to mild cognitive impairment. *Arch Neurol*. 2007;64(4):570-5.
64. MacKnight C, Rockwood K, Awalt E, McDowell I. Diabetes mellitus and the risk of dementia, Alzheimer's disease and vascular cognitive impairment in the Canadian Study of Health and Aging. *Dement Geriatr Cogn Disord*. 2002;14(2):77-83.
65. 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(9):1937-42.
66. Ravona-Springer R, Moshier E, Schmeidler J, Godbold J, Akrivos J, Rapp M, et al. Changes in glycemic control are associated with changes in cognition in non-diabetic elderly. *J Alzheimers Dis*. 2012;30(2):299-309.
67. Schrijvers EM, Witteman JC, Sijbrands EJ, Hofman A, Koudstaal PJ, Breteler MM. Insulin metabolism and the risk of Alzheimer disease: the Rotterdam Study. *Neurology*. 2010;75(22):1982-7.
68. Wu JH, Haan MN, Liang J, Ghosh D, Gonzalez HM, Herman WH. Impact of diabetes on cognitive function among older Latinos: a population-based cohort study. *J Clin Epidemiol*. 2003;56(7):686-93.
69. Wu JH, Haan MN, Liang J, Ghosh D, Gonzalez HM, Herman WH. Impact of antidiabetic medications on physical and cognitive functioning of older Mexican Americans with diabetes mellitus: a population-based cohort study. *Ann Epidemiol*. 2003;13(5):369-76.

70. Wu JH, Haan MN, Liang J, Ghosh D, Gonzalez HM, Herman WH. Diabetes as a predictor of change in functional status among older Mexican Americans: a population-based cohort study. *Diabetes Care*. 2003;26(2):314-9.
71. Bucht G, Adolfsson R, Lithner F, Winblad B. Changes in blood glucose and insulin secretion in patients with senile dementia of Alzheimer type. *Acta Med Scand*. 1983;213(5):387-92.
72. de la Monte SM. Early intranasal insulin therapy halts progression of neurodegeneration: progress in Alzheimer's disease therapeutics. *Aging health*. 2012;8(1):61-4.
73. Arnold SE, Arvanitakis Z, Macauley-Rambach SL, Koenig AM, Wang HY, Ahima RS, et al. Brain insulin resistance in type 2 diabetes and Alzheimer disease: concepts and conundrums. *Nat Rev Neurol*. 2018;14(3):168-81.
74. Kimura N. Diabetes Mellitus Induces Alzheimer's Disease Pathology: Histopathological Evidence from Animal Models. *Int J Mol Sci*. 2016;17(4):503.
75. Rotermund C, Truckenmuller FM, Schell H, Kahle PJ. Diet-induced obesity accelerates the onset of terminal phenotypes in alpha-synuclein transgenic mice. *J Neurochem*. 2014;131(6):848-58.
76. van Harten B, de Leeuw FE, Weinstein HC, Scheltens P, Biessels GJ. Brain imaging in patients with diabetes: a systematic review. *Diabetes Care*. 2006;29(11):2539-48.
77. den Heijer T, Vermeer SE, van Dijk EJ, Prins ND, Koudstaal PJ, Hofman A, et al. Type 2 diabetes and atrophy of medial temporal lobe structures on brain MRI. *Diabetologia*. 2003;46(12):1604-10.
78. Gold SM, Dziobek I, Sweat V, Tirsi A, Rogers K, Bruehl H, et al. Hippocampal damage and memory impairments as possible early brain complications of type 2 diabetes. *Diabetologia*. 2007;50(4):711-9.
79. Benedict C, Brooks SJ, Kullberg J, Burgos J, Kempton MJ, Nordenskjold R, et al. Impaired insulin sensitivity as indexed by the HOMA score is associated with deficits in verbal fluency and temporal lobe gray matter volume in the elderly. *Diabetes Care*. 2012;35(3):488-94.
80. Tan ZS, Beiser AS, Fox CS, Au R, Himali JJ, Debette S, et al. Association of metabolic dysregulation with volumetric brain magnetic resonance imaging and cognitive markers of subclinical brain aging in middle-aged adults: the Framingham Offspring Study. *Diabetes Care*. 2011;34(8):1766-70.

81. Rasgon NL, Kenna HA, Wroolie TE, Kelley R, Silverman D, Brooks J, et al. Insulin resistance and hippocampal volume in women at risk for Alzheimer's disease. *Neurobiol Aging*. 2011;32(11):1942-8.
82. Willette AA, Xu G, Johnson SC, Birdsill AC, Jonaitis EM, Sager MA, et al. Insulin resistance, brain atrophy, and cognitive performance in late middle-aged adults. *Diabetes Care*. 2013;36(2):443-9.
83. Hsu FC, Yuan M, Bowden DW, Xu J, Smith SC, Wagenknecht LE, et al. Adiposity is inversely associated with hippocampal volume in African Americans and European Americans with diabetes. *J Diabetes Complications*. 2016;30(8):1506-12.
84. Zhang YW, Zhang JQ, Liu C, Wei P, Zhang X, Yuan QY, et al. Memory dysfunction in type 2 diabetes mellitus correlates with reduced hippocampal CA1 and subiculum volumes. *Chin Med J (Engl)*. 2015;128(4):465-71.
85. Chen Y, Deng Y, Zhang B, Gong CX. Deregulation of brain insulin signaling in Alzheimer's disease. *Neurosci Bull*. 2014;30(2):282-94.
86. Hempel R, Onopa R, Convit A. Type 2 diabetes affects hippocampus volume differentially in men and women. *Diabetes Metab Res Rev*. 2012;28(1):76-83.
87. Moran C, Beare R, Phan TG, Bruce DG, Callisaya ML, Srikanth V, et al. Type 2 diabetes mellitus and biomarkers of neurodegeneration. *Neurology*. 2015;85(13):1123-30.
88. Brundel M, van den Berg E, Reijmer YD, de Bresser J, Kappelle LJ, Biessels GJ, et al. Cerebral haemodynamics, cognition and brain volumes in patients with type 2 diabetes. *J Diabetes Complications*. 2012;26(3):205-9.
89. Musen G, Jacobson AM, Bolo NR, Simonson DC, Shenton ME, McCartney RL, et al. Resting-state brain functional connectivity is altered in type 2 diabetes. *Diabetes*. 2012;61(9):2375-9.
90. Cui Y, Liang X, Gu H, Hu Y, Zhao Z, Yang XY, et al. Cerebral perfusion alterations in type 2 diabetes and its relation to insulin resistance and cognitive dysfunction. *Brain Imaging Behav*. 2017;11(5):1248-57.
91. Hoscheidt SM, Kellawan JM, Berman SE, Rivera-Rivera LA, Krause RA, Oh JM, et al. Insulin resistance is associated with lower arterial blood flow and reduced cortical perfusion in cognitively asymptomatic middle-aged adults. *J Cereb Blood Flow Metab*. 2017;37(6):2249-61.

92. Xia W, Rao H, Spaeth AM, Huang R, Tian S, Cai R, et al. Blood Pressure is Associated With Cerebral Blood Flow Alterations in Patients With T2DM as Revealed by Perfusion Functional MRI. *Medicine (Baltimore)*. 2015;94(48):e2231.
93. Xia W, Wang S, Rao H, Spaeth AM, Wang P, Yang Y, et al. Disrupted resting-state attentional networks in T2DM patients. *Sci Rep*. 2015;5:11148.
94. Ahtiluoto S, Polvikoski T, Peltonen M, Solomon A, Tuomilehto J, Winblad B, et al. Diabetes, Alzheimer disease, and vascular dementia: a population-based neuropathologic study. *Neurology*. 2010;75(13):1195-202.
95. Peila R, Rodriguez BL, Launer LJ, Honolulu-Asia Aging S. Type 2 diabetes, APOE gene, and the risk for dementia and related pathologies: The Honolulu-Asia Aging Study. *Diabetes*. 2002;51(4):1256-62.
96. Heitner J, Dickson D. Diabetics do not have increased Alzheimer-type pathology compared with age-matched control subjects. A retrospective postmortem immunocytochemical and histofluorescent study. *Neurology*. 1997;49(5):1306-11.
97. Beeri MS, Schmeidler J, Silverman JM, Gandy S, Wysocki M, Hannigan CM, et al. Insulin in combination with other diabetes medication is associated with less Alzheimer neuropathology. *Neurology*. 2008;71(10):750-7.
98. Arvanitakis Z, Schneider JA, Wilson RS, Li Y, Arnold SE, Wang Z, et al. Diabetes is related to cerebral infarction but not to AD pathology in older persons. *Neurology*. 2006;67(11):1960-5.
99. Thambisetty M, Jeffrey Metter E, Yang A, Dolan H, Marano C, Zonderman AB, et al. Glucose intolerance, insulin resistance, and pathological features of Alzheimer disease in the Baltimore Longitudinal Study of Aging. *JAMA Neurol*. 2013;70(9):1167-72.
100. Roberts RO, Knopman DS, Cha RH, Mielke MM, Pankratz VS, Boeve BF, et al. Diabetes and elevated hemoglobin A1c levels are associated with brain hypometabolism but not amyloid accumulation. *J Nucl Med*. 2014;55(5):759-64.
101. Tomita N, Furukawa K, Okamura N, Tashiro M, Une K, Furumoto S, et al. Brain accumulation of amyloid beta protein visualized by positron emission tomography and BF-227 in Alzheimer's disease patients with or without diabetes mellitus. *Geriatr Gerontol Int*. 2013;13(1):215-21.

102. Fukasawa R, Hanyu H, Shimizu S, Kanetaka H, Sakurai H, Ishii K. Identification of diabetes-related dementia: Longitudinal perfusion SPECT and amyloid PET studies. *J Neurol Sci.* 2015;349(1-2):45-51.
103. Bateman RJ, Xiong C, Benzinger TL, Fagan AM, Goate A, Fox NC, et al. Clinical and biomarker changes in dominantly inherited Alzheimer's disease. *N Engl J Med.* 2012;367(9):795-804.
104. Janson J, Laedtke T, Parisi JE, O'Brien P, Petersen RC, Butler PC. Increased risk of type 2 diabetes in Alzheimer disease. *Diabetes.* 2004;53(2):474-81.
105. Knight EM, Ruiz HH, Kim SH, Harte JC, Hsieh W, Glabe C, et al. Unexpected partial correction of metabolic and behavioral phenotypes of Alzheimer's APP/PSEN1 mice by gene targeting of diabetes/Alzheimer's-related Sorcs1. *Acta Neuropathol Commun.* 2016;4:16.
106. Ng TP, Feng L, Nyunt MS, Feng L, Gao Q, Lim ML, et al. Metabolic Syndrome and the Risk of Mild Cognitive Impairment and Progression to Dementia: Follow-up of the Singapore Longitudinal Ageing Study Cohort. *JAMA Neurol.* 2016;73(4):456-63.
107. Mehla J, Chauhan BC, Chauhan NB. Experimental induction of type 2 diabetes in aging-accelerated mice triggered Alzheimer-like pathology and memory deficits. *J Alzheimers Dis.* 2014;39(1):145-62.
108. Julien C, Tremblay C, Phivilay A, Berthiaume L, Emond V, Julien P, et al. High-fat diet aggravates amyloid-beta and tau pathologies in the 3xTg-AD mouse model. *Neurobiol Aging.* 2010;31(9):1516-31.
109. Mouton PR, Chachich ME, Quigley C, Spangler E, Ingram DK. Caloric restriction attenuates amyloid deposition in middle-aged dtg APP/PS1 mice. *Neurosci Lett.* 2009;464(3):184-7.
110. de Nazareth AM. Type 2 diabetes mellitus in the pathophysiology of Alzheimer's disease. *Dement Neuropsychol.* 2017;11(2):105-13.
111. Bosco D, Fava A, Plastino M, Montalcini T, Pujia A. Possible implications of insulin resistance and glucose metabolism in Alzheimer's disease pathogenesis. *J Cell Mol Med.* 2011;15(9):1807-21.
112. Adlerz L, Holback S, Multhaup G, Iverfeldt K. IGF-1-induced processing of the amyloid precursor protein family is mediated by different signaling pathways. *J Biol Chem.* 2007;282(14):10203-9.

113. Yarchoan M, Arnold SE. Repurposing diabetes drugs for brain insulin resistance in Alzheimer disease. *Diabetes*. 2014;63(7):2253-61.
114. Bomfim TR, Forny-Germano L, Sathler LB, Brito-Moreira J, Houzel JC, Decker H, et al. An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease- associated Abeta oligomers. *J Clin Invest*. 2012;122(4):1339-53.
115. Craft S, Asthana S, Cook DG, Baker LD, Cherrier M, Purganan K, et al. Insulin dose-response effects on memory and plasma amyloid precursor protein in Alzheimer's disease: interactions with apolipoprotein E genotype. *Psychoneuroendocrinology*. 2003;28(6):809-22.
116. Boyt AA, Taddei TK, Hallmayer J, Helmerhorst E, Gandy SE, Craft S, et al. The effect of insulin and glucose on the plasma concentration of Alzheimer's amyloid precursor protein. *Neuroscience*. 2000;95(3):727-34.
117. Talbot K, Wang HY, Kazi H, Han LY, Bakshi KP, Stucky A, et al. Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *J Clin Invest*. 2012;122(4):1316-38.
118. Hiltunen M, Khandelwal VK, Yaluri N, Tiilikainen T, Tusa M, Koivisto H, et al. Contribution of genetic and dietary insulin resistance to Alzheimer phenotype in APP/PS1 transgenic mice. *J Cell Mol Med*. 2012;16(6):1206-22.
119. Kullmann S, Heni M, Hallschmid M, Fritsche A, Preissl H, Haring HU. Brain Insulin Resistance at the Crossroads of Metabolic and Cognitive Disorders in Humans. *Physiol Rev*. 2016;96(4):1169-209.
120. Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, 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(1):63-80.
121. Frolich L, Blum-Degen D, Bernstein HG, Engelsberger S, Humrich J, Laufer S, et al. Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. *J Neural Transm (Vienna)*. 1998;105(4-5):423-38.
122. Rivera EJ, Goldin A, Fulmer N, Tavares R, Wands JR, de la Monte SM. Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: link to brain reductions in acetylcholine. *J Alzheimers Dis*. 2005;8(3):247-68.

123. Moloney AM, Griffin RJ, Timmons S, O'Connor R, Ravid R, O'Neill C. Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling. *Neurobiol Aging*. 2010;31(2):224-43.
124. Frolich L, Blum-Degen D, Riederer P, Hoyer S. A disturbance in the neuronal insulin receptor signal transduction in sporadic Alzheimer's disease. *Ann N Y Acad Sci*. 1999;893:290-3.
125. Folch J, Ettcheto M, Busquets O, Sanchez-Lopez E, Castro-Torres RD, Verdaguer E, et al. The Implication of the Brain Insulin Receptor in Late Onset Alzheimer's Disease Dementia. *Pharmaceuticals (Basel)*. 2018;11(1).
126. Biessels GJ, Reagan LP. Hippocampal insulin resistance and cognitive dysfunction. *Nat Rev Neurosci*. 2015;16(11):660-71.
127. McNay EC, Recknagel AK. Brain insulin signaling: a key component of cognitive processes and a potential basis for cognitive impairment in type 2 diabetes. *Neurobiol Learn Mem*. 2011;96(3):432-42.
128. Schioth HB, Craft S, Brooks SJ, Frey WH, 2nd, Benedict C. Brain insulin signaling and Alzheimer's disease: current evidence and future directions. *Mol Neurobiol*. 2012;46(1):4-10.
129. Pearson-Leary J, McNay EC. Intrahippocampal administration of amyloid-beta(1-42) oligomers acutely impairs spatial working memory, insulin signaling, and hippocampal metabolism. *J Alzheimers Dis*. 2012;30(2):413-22.
130. Walker JM, Harrison FE. Shared Neuropathological Characteristics of Obesity, Type 2 Diabetes and Alzheimer's Disease: Impacts on Cognitive Decline. *Nutrients*. 2015;7(9):7332-57.
131. Solano DC, Sironi M, Bonfini C, Solerte SB, Govoni S, Racchi M. Insulin regulates soluble amyloid precursor protein release via phosphatidyl inositol 3 kinase-dependent pathway. *FASEB J*. 2000;14(7):1015-22.
132. Gasparini L, Gouras GK, Wang R, Gross RS, Beal MF, Greengard P, et al. Stimulation of beta-amyloid precursor protein trafficking by insulin reduces intraneuronal beta-amyloid and requires mitogen-activated protein kinase signaling. *J Neurosci*. 2001;21(8):2561-70.
133. Ashpole NM, Sanders JE, Hodges EL, Yan H, Sonntag WE. Growth hormone, insulin-like growth factor-1 and the aging brain. *Exp Gerontol*. 2015;68:76-81.

134. Zhao L, Teter B, Morihara T, Lim GP, Ambegaokar SS, Ubeda OJ, et al. Insulin-degrading enzyme as a downstream target of insulin receptor signaling cascade: implications for Alzheimer's disease intervention. *J Neurosci*. 2004;24(49):11120-6.
135. Xie L, Helmerhorst E, Taddei K, Plewright B, Van Bronswijk W, Martins R. Alzheimer's beta-amyloid peptides compete for insulin binding to the insulin receptor. *J Neurosci*. 2002;22(10):RC221.
136. Tharp WG, Gupta D, Smith J, Jones KP, Jones AM, Pratley RE. Effects of glucose and insulin on secretion of amyloid-beta by human adipose tissue cells. *Obesity (Silver Spring)*. 2016;24(7):1471-9.
137. Zhao WQ, De Felice FG, Fernandez S, Chen H, Lambert MP, Quon MJ, et al. Amyloid beta oligomers induce impairment of neuronal insulin receptors. *FASEB J*. 2008;22(1):246-60.
138. De Felice FG, Vieira MN, Bomfim TR, Decker H, Velasco PT, Lambert MP, et al. Protection of synapses against Alzheimer's-linked toxins: insulin signaling prevents the pathogenic binding of Abeta oligomers. *Proc Natl Acad Sci U S A*. 2009;106(6):1971-6.
139. De Felice FG. Alzheimer's disease and insulin resistance: translating basic science into clinical applications. *J Clin Invest*. 2013;123(2):531-9.
140. Avgerinos KI, Kalaitzidis G, Malli A, Kalaitzoglou D, Myserlis PG, Lioutas VA. Intranasal insulin in Alzheimer's dementia or mild cognitive impairment: a systematic review. *J Neurol*. 2018;265(7):1497-510.
141. Landau SM, Harvey D, Madison CM, Koeppe RA, Reiman EM, Foster NL, et al. Associations between cognitive, functional, and FDG-PET measures of decline in AD and MCI. *Neurobiol Aging*. 2011;32(7):1207-18.
142. Rizvi SM, Shaikh S, Waseem SM, Shakil S, Abuzenadah AM, Biswas D, et al. Role of anti-diabetic drugs as therapeutic agents in Alzheimer's disease. *EXCLI J*. 2015;14:684-96.
143. Bedse G, Di Domenico F, Serviddio G, Cassano T. Aberrant insulin signaling in Alzheimer's disease: current knowledge. *Front Neurosci*. 2015;9:204.
144. Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. *J Clin Invest*. 2001;108(6):779-84.
145. Herz J, Hamann U, Rogne S, Myklebost O, Gausepohl H, Stanley KK. Surface location and high affinity for calcium of a 500-kd liver membrane protein

closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO J.* 1988;7(13):4119-27.

146. Bu G. Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy. *Nat Rev Neurosci.* 2009;10(5):333-44.

147. Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK. LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. *Physiol Rev.* 2008;88(3):887-918.

148. Boucher P, Herz J. Signaling through LRP1: Protection from atherosclerosis and beyond. *Biochem Pharmacol.* 2011;81(1):1-5.

149. Spuch C, Ortolano S, Navarro C. LRP-1 and LRP-2 receptors function in the membrane neuron. Trafficking mechanisms and proteolytic processing in Alzheimer's disease. *Front Physiol.* 2012;3:269.

150. Lin L, Hu K. LRP-1: functions, signaling and implications in kidney and other diseases. *Int J Mol Sci.* 2014;15(12):22887-901.

151. Strickland DK, Ranganathan S. Diverse role of LDL receptor-related protein in the clearance of proteases and in signaling. *J Thromb Haemost.* 2003;1(7):1663-70.

152. van Kerkhof P, Lee J, McCormick L, Tetrault E, Lu W, Schoenfish M, et al. Sorting nexin 17 facilitates LRP recycling in the early endosome. *EMBO J.* 2005;24(16):2851-61.

153. Li Y, Marzolo MP, van Kerkhof P, Strous GJ, Bu G. The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. *J Biol Chem.* 2000;275(22):17187-94.

154. Hayashi H, Campenot RB, Vance DE, Vance JE. Apolipoprotein E-containing lipoproteins protect neurons from apoptosis via a signaling pathway involving low-density lipoprotein receptor-related protein-1. *J Neurosci.* 2007;27(8):1933-41.

155. Hu K, Yang J, Tanaka S, Gonias SL, Mars WM, Liu Y. Tissue-type plasminogen activator acts as a cytokine that triggers intracellular signal transduction and induces matrix metalloproteinase-9 gene expression. *J Biol Chem.* 2006;281(4):2120-7.

156. Mantuano E, Inoue G, Li X, Takahashi K, Gaultier A, Gonias SL, et al. The hemopexin domain of matrix metalloproteinase-9 activates cell signaling and promotes migration of schwann cells by binding to low-density lipoprotein receptor-related protein. *J Neurosci.* 2008;28(45):11571-82.

157. Shi Y, Mantuano E, Inoue G, Campana WM, Gonias SL. Ligand binding to LRP1 transactivates Trk receptors by a Src family kinase-dependent pathway. *Sci Signal*. 2009;2(68):ra18.
158. May P, Reddy YK, Herz J. Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. *J Biol Chem*. 2002;277(21):18736-43.
159. Liu CC, Hu J, Tsai CW, Yue M, Melrose HL, Kanekiyo T, et al. Neuronal LRP1 regulates glucose metabolism and insulin signaling in the brain. *J Neurosci*. 2015;35(14):5851-9.
160. Fuentealba RA, Liu Q, Kanekiyo T, Zhang J, Bu G. Low density lipoprotein receptor-related protein 1 promotes anti-apoptotic signaling in neurons by activating Akt survival pathway. *J Biol Chem*. 2009;284(49):34045-53.
161. Liu Q, Trotter J, Zhang J, Peters MM, Cheng H, Bao J, et al. Neuronal LRP1 knockout in adult mice leads to impaired brain lipid metabolism and progressive, age-dependent synapse loss and neurodegeneration. *J Neurosci*. 2010;30(50):17068-78.
162. Jones AR, Shusta EV. Blood-brain barrier transport of therapeutics via receptor-mediation. *Pharm Res*. 2007;24(9):1759-71.
163. Sweeney MD, Sagare AP, Zlokovic BV. Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nat Rev Neurol*. 2018;14(3):133-50.
164. Wang YY, Lui PC, Li JY. Receptor-mediated therapeutic transport across the blood-brain barrier. *Immunotherapy*. 2009;1(6):983-93.
165. Deane R, Bell RD, Sagare A, Zlokovic BV. Clearance of amyloid-beta peptide across the blood-brain barrier: implication for therapies in Alzheimer's disease. *CNS Neurol Disord Drug Targets*. 2009;8(1):16-30.
166. Hultman K, Strickland S, Norris EH. The APOE varepsilon4/varepsilon4 genotype potentiates vascular fibrin(ogen) deposition in amyloid-laden vessels in the brains of Alzheimer's disease patients. *J Cereb Blood Flow Metab*. 2013;33(8):1251-8.
167. Zipser BD, Johanson CE, Gonzalez L, Berzin TM, Tavares R, Hulette CM, et al. Microvascular injury and blood-brain barrier leakage in Alzheimer's disease. *Neurobiol Aging*. 2007;28(7):977-86.

168. Ryu JK, McLarnon JG. A leaky blood-brain barrier, fibrinogen infiltration and microglial reactivity in inflamed Alzheimer's disease brain. *J Cell Mol Med.* 2009;13(9A):2911-25.
169. Cortes-Canteli M, Paul J, Norris EH, Bronstein R, Ahn HJ, Zamolodchikov D, et al. Fibrinogen and beta-amyloid association alters thrombosis and fibrinolysis: a possible contributing factor to Alzheimer's disease. *Neuron.* 2010;66(5):695-709.
170. Sengillo JD, Winkler EA, Walker CT, Sullivan JS, Johnson M, Zlokovic BV. Deficiency in mural vascular cells coincides with blood-brain barrier disruption in Alzheimer's disease. *Brain Pathol.* 2013;23(3):303-10.
171. Halliday MR, Rege SV, Ma Q, Zhao Z, Miller CA, Winkler EA, et al. Accelerated pericyte degeneration and blood-brain barrier breakdown in apolipoprotein E4 carriers with Alzheimer's disease. *J Cereb Blood Flow Metab.* 2016;36(1):216-27.
172. 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(11 Suppl 1):2628-31.
173. Zlokovic BV, Martel CL, Matsubara E, McComb JG, Zheng G, McCluskey RT, et al. Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid beta at the blood-brain and blood-cerebrospinal fluid barriers. *Proc Natl Acad Sci U S A.* 1996;93(9):4229-34.
174. Yamada M. Cerebral amyloid angiopathy: emerging concepts. *J Stroke.* 2015;17(1):17-30.
175. Biffi A, Greenberg SM. Cerebral amyloid angiopathy: a systematic review. *J Clin Neurol.* 2011;7(1):1-9.
176. Krohn M, Lange C, Hofrichter J, Scheffler K, Stenzel J, Steffen J, et al. Cerebral amyloid-beta proteostasis is regulated by the membrane transport protein ABCC1 in mice. *J Clin Invest.* 2011;121(10):3924-31.
177. Cirrito JR, Deane R, Fagan AM, Spinner ML, Parsadanian M, Finn MB, et al. P-glycoprotein deficiency at the blood-brain barrier increases amyloid-beta deposition in an Alzheimer disease mouse model. *J Clin Invest.* 2005;115(11):3285-90.
178. Koldamova R, Fitz NF, Lefterov I. ATP-binding cassette transporter A1: from metabolism to neurodegeneration. *Neurobiol Dis.* 2014;72 Pt A:13-21.

179. Gonda DK, Bachmair A, Wunning I, Tobias JW, Lane WS, Varshavsky A. Universality and structure of the N-end rule. *J Biol Chem*. 1989;264(28):16700-12.
180. Bachmair A, Finley D, Varshavsky A. In vivo half-life of a protein is a function of its amino-terminal residue. *Science*. 1986;234(4773):179-86.
181. Varshavsky A. The N-end rule pathway of protein degradation. *Genes Cells*. 1997;2(1):13-28.
182. Gregori L, Fuchs C, Figueiredo-Pereira ME, Van Nostrand WE, Goldgaber D. Amyloid beta-protein inhibits ubiquitin-dependent protein degradation in vitro. *J Biol Chem*. 1995;270(34):19702-8.
183. Gregori L, Hainfeld JF, Simon MN, Goldgaber D. Binding of amyloid beta protein to the 20 S proteasome. *J Biol Chem*. 1997;272(1):58-62.
184. Marambaud P, Chevallier N, Barelli H, Wilk S, Checler F. Proteasome contributes to the alpha-secretase pathway of amyloid precursor protein in human cells. *J Neurochem*. 1997;68(2):698-703.
185. Marambaud P, Lopez-Perez E, Wilk S, Checler F. Constitutive and protein kinase C-regulated secretory cleavage of Alzheimer's beta-amyloid precursor protein: different control of early and late events by the proteasome. *J Neurochem*. 1997;69(6):2500-5.
186. Baranello RJ, Bharani KL, Padmaraju V, Chopra N, Lahiri DK, Greig NH, et al. Amyloid-beta protein clearance and degradation (ABCD) pathways and their role in Alzheimer's disease. *Curr Alzheimer Res*. 2015;12(1):32-46.
187. Boland B, Kumar A, Lee S, Platt FM, Wegiel J, Yu WH, et al. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *J Neurosci*. 2008;28(27):6926-37.
188. Baehrecke EH. Autophagy: dual roles in life and death? *Nat Rev Mol Cell Biol*. 2005;6(6):505-10.
189. Nakanishi H, Amano T, Sastradipura DF, Yoshimine Y, Tsukuba T, Tanabe K, et al. Increased expression of cathepsins E and D in neurons of the aged rat brain and their colocalization with lipofuscin and carboxy-terminal fragments of Alzheimer amyloid precursor protein. *J Neurochem*. 1997;68(2):739-49.
190. Cataldo AM, Barnett JL, Berman SA, Li J, Quarless S, Bursztajn S, et al. Gene expression and cellular content of cathepsin D in Alzheimer's disease brain: evidence for early up-regulation of the endosomal-lysosomal system. *Neuron*. 1995;14(3):671-80.

191. Nixon RA, Cataldo AM. The lysosomal system in neuronal cell death: a review. *Ann N Y Acad Sci.* 1993;679:87-109.
192. Li J, Kanekiyo T, Shinohara M, Zhang Y, LaDu MJ, Xu H, et al. Differential regulation of amyloid-beta endocytic trafficking and lysosomal degradation by apolipoprotein E isoforms. *J Biol Chem.* 2012;287(53):44593-601.
193. Cataldo AM, Barnett JL, Pieroni C, Nixon RA. Increased neuronal endocytosis and protease delivery to early endosomes in sporadic Alzheimer's disease: neuropathologic evidence for a mechanism of increased beta-amyloidogenesis. *J Neurosci.* 1997;17(16):6142-51.
194. Bustamante HA, Gonzalez AE, Cerda-Troncoso C, Shaughnessy R, Otth C, Soza A, et al. Interplay Between the Autophagy-Lysosomal Pathway and the Ubiquitin-Proteasome System: A Target for Therapeutic Development in Alzheimer's Disease. *Front Cell Neurosci.* 2018;12:126.
195. Velazquez R, Tran A, Ishimwe E, Denner L, Dave N, Oddo S, et al. Central insulin dysregulation and energy dyshomeostasis in two mouse models of Alzheimer's disease. *Neurobiol Aging.* 2017;58:1-13.
196. Kober AC, Manavalan APC, Tam-Amersdorfer C, Holmer A, Saeed A, Fanaee-Danesh E, et al. Implications of cerebrovascular ATP-binding cassette transporter G1 (ABCG1) and apolipoprotein M in cholesterol transport at the blood-brain barrier. *Biochim Biophys Acta.* 2017;1862(6):573-88.
197. Zandi-Lang M, Fanaee-Danesh E, Sun Y, Albrecher NM, Gali CC, Cancar I, et al. Regulatory effects of simvastatin and apoJ on APP processing and amyloid-beta clearance in blood-brain barrier endothelial cells. *Biochim Biophys Acta.* 2018;1863(1):40-60.
198. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods.* 2001;25(4):402-8.
199. Zenaro E, Pietronigro E, Della Bianca V, Piacentino G, Marongiu L, Budui S, et al. Neutrophils promote Alzheimer's disease-like pathology and cognitive decline via LFA-1 integrin. *Nat Med.* 2015;21(8):880-6.
200. Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kaye R, et al. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron.* 2003;39(3):409-21.

201. Attar A, Liu T, Chan WT, Hayes J, Nejad M, Lei K, et al. A shortened Barnes maze protocol reveals memory deficits at 4-months of age in the triple-transgenic mouse model of Alzheimer's disease. *PLoS One*. 2013;8(11):e80355.
202. Casali BT, Landreth GE. Abeta Extraction from Murine Brain Homogenates. *Bio Protoc*. 2016;6(8).
203. Chirackal Manavalan AP, Kober A, Metso J, Lang I, Becker T, Hasslitz K, et al. Phospholipid transfer protein is expressed in cerebrovascular endothelial cells and involved in high density lipoprotein biogenesis and remodeling at the blood-brain barrier. *J Biol Chem*. 2014;289(8):4683-98.
204. Churukian CJ. Improved Puchtler's Congo red method for demonstrating amyloid. *J Histotechnol*. 2000;23(2):139-41.
205. Yang GY, Xu B, Hashimoto T, Huey M, Chaly T, Jr., Wen R, et al. Induction of focal angiogenesis through adenoviral vector mediated vascular endothelial cell growth factor gene transfer in the mature mouse brain. *Angiogenesis*. 2003;6(2):151-8.
206. Vandal M, White PJ, Chevrier G, Tremblay C, St-Amour I, Planel E, et al. Age-dependent impairment of glucose tolerance in the 3xTg-AD mouse model of Alzheimer's disease. *FASEB J*. 2015;29(10):4273-84.
207. Vandal M, White PJ, Tremblay C, St-Amour I, Chevrier G, Emond V, et al. Insulin reverses the high-fat diet-induced increase in brain Abeta and improves memory in an animal model of Alzheimer disease. *Diabetes*. 2014;63(12):4291-301.
208. Tamaki C, Ohtsuki S, Iwatsubo T, Hashimoto T, Yamada K, Yabuki C, et al. Major involvement of low-density lipoprotein receptor-related protein 1 in the clearance of plasma free amyloid beta-peptide by the liver. *Pharm Res*. 2006;23(7):1407-16.
209. Tamaki C, Ohtsuki S, Terasaki T. Insulin facilitates the hepatic clearance of plasma amyloid beta-peptide (1-40) by intracellular translocation of low-density lipoprotein receptor-related protein 1 (LRP-1) to the plasma membrane in hepatocytes. *Mol Pharmacol*. 2007;72(4):850-5.
210. Deane R, Wu Z, Sagare A, Davis J, Du Yan S, Hamm K, et al. LRP/amyloid beta-peptide interaction mediates differential brain efflux of Abeta isoforms. *Neuron*. 2004;43(3):333-44.

211. Storck SE, Hartz AMS, Bernard J, Wolf A, Kachlmeier A, Mahringer A, et al. The concerted amyloid-beta clearance of LRP1 and ABCB1/P-gp across the blood-brain barrier is linked by PICALM. *Brain Behav Immun*. 2018;73:21-33.
212. Storck SE, Meister S, Nahrath J, Meissner JN, Schubert N, Di Spiezio A, et al. Endothelial LRP1 transports amyloid-beta(1-42) across the blood-brain barrier. *J Clin Invest*. 2016;126(1):123-36.
213. Kachooei E, Moosavi-Movahedi AA, Khodagholi F, Ramshini H, Shaerzadeh F, Sheibani N. Oligomeric forms of insulin amyloid aggregation disrupt outgrowth and complexity of neuron-like PC12 cells. *PLoS One*. 2012;7(7):e41344.
214. Kirouac L, Rajic AJ, Cribbs DH, Padmanabhan J. Activation of Ras-ERK Signaling and GSK-3 by Amyloid Precursor Protein and Amyloid Beta Facilitates Neurodegeneration in Alzheimer's Disease. *eNeuro*. 2017;4(2).
215. Matsuzaki T, Sasaki K, Tanizaki Y, Hata J, Fujimi K, Matsui Y, et al. Insulin resistance is associated with the pathology of Alzheimer disease: the Hisayama study. *Neurology*. 2010;75(9):764-70.
216. Ong QR, Chan ES, Lim ML, Cole GM, Wong BS. Reduced phosphorylation of brain insulin receptor substrate and Akt proteins in apolipoprotein-E4 targeted replacement mice. *Sci Rep*. 2014;4:3754.
217. Craft S, Baker LD, Montine TJ, Minoshima S, Watson GS, Claxton A, et al. Intranasal insulin therapy for Alzheimer disease and amnesic mild cognitive impairment: a pilot clinical trial. *Arch Neurol*. 2012;69(1):29-38.
218. Cao B, Rosenblat JD, Brietzke E, Park C, Lee Y, Musial N, et al. Comparative efficacy and acceptability of antidiabetic agents for Alzheimer's disease and mild cognitive impairment: A systematic review and network meta-analysis. *Diabetes Obes Metab*. 2018;20(10):2467-71.
219. Alagiakrishnan K, Sankaralingam S, Ghosh M, Mereu L, Senior P. Antidiabetic drugs and their potential role in treating mild cognitive impairment and Alzheimer's disease. *Discov Med*. 2013;16(90):277-86.
220. Yang Y, Smith DL, Jr., Keating KD, Allison DB, Nagy TR. Variations in body weight, food intake and body composition after long-term high-fat diet feeding in C57BL/6J mice. *Obesity (Silver Spring)*. 2014;22(10):2147-55.
221. Pettersson US, Walden TB, Carlsson PO, Jansson L, Phillipson M. Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue. *PLoS One*. 2012;7(9):e46057.

222. Hirata-Fukae C, Li HF, Hoe HS, Gray AJ, Minami SS, Hamada K, et al. Females exhibit more extensive amyloid, but not tau, pathology in an Alzheimer transgenic model. *Brain Res.* 2008;1216:92-103.
223. Carroll JC, Rosario ER, Kreimer S, Villamagna A, Gentzschein E, Stanczyk FZ, et al. Sex differences in beta-amyloid accumulation in 3xTg-AD mice: role of neonatal sex steroid hormone exposure. *Brain Res.* 2010;1366:233-45.
224. Paruthiyil S, Hagiwara SI, Kundassery K, Bhargava A. Sexually dimorphic metabolic responses mediated by CRF2 receptor during nutritional stress in mice. *Biol Sex Differ.* 2018;9(1):49.
225. Nagga K, Gustavsson AM, Stomrud E, Lindqvist D, van Westen D, Blennow K, et al. Increased midlife triglycerides predict brain beta-amyloid and tau pathology 20 years later. *Neurology.* 2018;90(1):e73-e81.
226. Burgess BL, McIsaac SA, Naus KE, Chan JY, Tansley GH, Yang J, et al. Elevated plasma triglyceride levels precede amyloid deposition in Alzheimer's disease mouse models with abundant A beta in plasma. *Neurobiol Dis.* 2006;24(1):114-27.
227. Wang X, Magkos F, Mittendorfer B. Sex differences in lipid and lipoprotein metabolism: it's not just about sex hormones. *J Clin Endocrinol Metab.* 2011;96(4):885-93.
228. Au DT, Strickland DK, Muratoglu SC. The LDL Receptor-Related Protein 1: At the Crossroads of Lipoprotein Metabolism and Insulin Signaling. *J Diabetes Res.* 2017;2017:8356537.
229. Liu Q, Zhang J, Zerbinatti C, Zhan Y, Kolber BJ, Herz J, et al. Lipoprotein receptor LRP1 regulates leptin signaling and energy homeostasis in the adult central nervous system. *PLoS Biol.* 2011;9(1):e1000575.
230. Borlikova GG, Trejo M, Mably AJ, McDonald JM, Sala Frigerio C, Regan CM, et al. Alzheimer brain-derived amyloid beta-protein impairs synaptic remodeling and memory consolidation. *Neurobiol Aging.* 2013;34(5):1315-27.
231. Xia W. Brain amyloid beta protein and memory disruption in Alzheimer's disease. *Neuropsychiatr Dis Treat.* 2010;6:605-11.
232. Faucher P, Mons N, Micheau J, Louis C, Beracochea DJ. Hippocampal Injections of Oligomeric Amyloid beta-peptide (1-42) Induce Selective Working Memory Deficits and Long-lasting Alterations of ERK Signaling Pathway. *Front Aging Neurosci.* 2015;7:245.

233. Swaminathan SK, Ahlschwede KM, Sarma V, Curran GL, Omtri RS, Decklever T, et al. Insulin differentially affects the distribution kinetics of amyloid beta 40 and 42 in plasma and brain. *J Cereb Blood Flow Metab.* 2017;271678X17709709.
234. Vekrellis K, Ye Z, Qiu WQ, Walsh D, Hartley D, Chesneau V, et al. Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme. *J Neurosci.* 2000;20(5):1657-65.
235. Farris W, Mansourian S, Chang Y, Lindsley L, Eckman EA, Frosch MP, et al. Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. *Proc Natl Acad Sci U S A.* 2003;100(7):4162-7.
236. Clinton LK, Billings LM, Green KN, Caccamo A, Ngo J, Oddo S, et al. Age-dependent sexual dimorphism in cognition and stress response in the 3xTg-AD mice. *Neurobiol Dis.* 2007;28(1):76-82.
237. Huang CC, Lee CC, Hsu KS. The role of insulin receptor signaling in synaptic plasticity and cognitive function. *Chang Gung Med J.* 2010;33(2):115-25.
238. De Felice FG, Benedict C. A Key Role of Insulin Receptors in Memory. *Diabetes.* 2015;64(11):3653-5.
239. Grillo CA, Piroli GG, Lawrence RC, Wrihten SA, Green AJ, Wilson SP, et al. Hippocampal Insulin Resistance Impairs Spatial Learning and Synaptic Plasticity. *Diabetes.* 2015;64(11):3927-36.
240. Su F, Shu H, Ye Q, Wang Z, Xie C, Yuan B, et al. Brain insulin resistance deteriorates cognition by altering the topological features of brain networks. *Neuroimage Clin.* 2017;13:280-7.
241. Kucejova B, Duarte J, Satapati S, Fu X, Ilkayeva O, Newgard CB, et al. Hepatic mTORC1 Opposes Impaired Insulin Action to Control Mitochondrial Metabolism in Obesity. *Cell Rep.* 2016;16(2):508-19.
242. Subramaniam S, Unsicker K. ERK and cell death: ERK1/2 in neuronal death. *FEBS J.* 2010;277(1):22-9.
243. Ghasemi R, Zarifkar A, Rastegar K, Maghsoudi N, Moosavi M. Repeated intra-hippocampal injection of beta-amyloid 25-35 induces a reproducible impairment of learning and memory: considering caspase-3 and MAPKs activity. *Eur J Pharmacol.* 2014;726:33-40.

244. Engelman JA, Berg AH, Lewis RY, Lisanti MP, Scherer PE. Tumor necrosis factor alpha-mediated insulin resistance, but not dedifferentiation, is abrogated by MEK1/2 inhibitors in 3T3-L1 adipocytes. *Mol Endocrinol*. 2000;14(10):1557-69.
245. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron*. 2008;57(2):178-201.
246. Montagne A, Zhao Z, Zlokovic BV. Alzheimer's disease: A matter of blood-brain barrier dysfunction? *J Exp Med*. 2017;214(11):3151-69.
247. Do TM, Dodacki A, Alata W, Calon F, Nicolic S, Scherrmann JM, et al. Age-Dependent Regulation of the Blood-Brain Barrier Influx/Efflux Equilibrium of Amyloid-beta Peptide in a Mouse Model of Alzheimer's Disease (3xTg-AD). *J Alzheimers Dis*. 2016;49(2):287-300.
248. Boland B, Yu WH, Corti O, Mollereau B, Henriques A, Bezard E, et al. Promoting the clearance of neurotoxic proteins in neurodegenerative disorders of ageing. *Nat Rev Drug Discov*. 2018;17(9):660-88.
249. Evrard C, Kienlen-Campard P, Coevoet M, Opsomer R, Tasiaux B, Melnyk P, et al. Contribution of the Endosomal-Lysosomal and Proteasomal Systems in Amyloid-beta Precursor Protein Derived Fragments Processing. *Front Cell Neurosci*. 2018;12:435.
250. Fonseca AC, Ferreiro E, Oliveira CR, Cardoso SM, Pereira CF. Activation of the endoplasmic reticulum stress response by the amyloid-beta 1-40 peptide in brain endothelial cells. *Biochim Biophys Acta*. 2013;1832(12):2191-203.
251. Krupp MN, Lane MD. Evidence for different pathways for the degradation of insulin and insulin receptor in the chick liver cell. *J Biol Chem*. 1982;257(3):1372-7.
252. Wujak L, Bottcher RT, Pak O, Frey H, El Agha E, Chen Y, et al. Low density lipoprotein receptor-related protein 1 couples beta1 integrin activation to degradation. *Cell Mol Life Sci*. 2018;75(9):1671-85.