Amyloid-beta impairs insulin signaling by accelerating autophagy-lysosomal degradation of LRP-1 and IR-β in blood-brain barrier endothelial cells \textit{in vitro} and in 3XTg-AD mice

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\textbf{A R T I C L E   I N F O}

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\textbf{ABSTRACT}

Aberrant insulin signaling constitutes an early change in Alzheimer's disease (AD). Insulin receptors (IR) and low-density lipoprotein receptor-related protein-1 (LRP-1) are expressed in brain capillary endothelial cells (BCEC) forming the blood-brain barrier (BBB). There, insulin may regulate the function of LRP-1 in Aβ clearance from the brain. Changes in IR-β and LRP-1 and insulin signaling at the BBB in AD are not well understood. Herein, we identified a reduction in cerebral and cerebrovascular IR-β levels in 9-month-old male and female 3XTg-AD (PS1\textsuperscript{M146V}, APP\textsuperscript{swen} and tauP301L) as compared to NTg mice, which is important in insulin mediated signaling responses. Reduced cerebral IR-β levels corresponded to impaired insulin signaling and LRP-1 levels in brain. Reduced cerebral and cerebrovascular IR-β and LRP-1 levels in 3XTg-AD mice correlated with elevated levels of autophagy marker LC3B. In both genotypes, high-fat diet (HFD) feeding decreased cerebral and hepatic LRP-1 expression and elevated cerebral Aβ burden without affecting cerebrovascular LRP-1 and IR-β levels. In \textit{in vitro} studies using primary porcine (p)BCEC revealed that Aβ peptides 1–40 or 1–42 (240 nM) reduced cellular levels and interaction of LRP-1 and IR-β thereby perturbing insulin-mediated signaling. Further mechanistic investigation revealed that Aβ treatment accelerated the autophagy-lysosomal degradation of IR-β and LRP-1 in pBCEC. LRP-1 silencing in pBCEC decreased IR-β levels through post-translational pathways further deteriorating insulin-mediated responses at the BBB. Our findings indicate that LRP-1 proves important for insulin signaling at the BBB. Cerebral Aβ burden in AD may accelerate LRP-1 and IR-β degradation in BCEC thereby contributing to impaired cerebral and cerebromicrovascular insulin effects.

\textit{Abbreviations:} 3XTg-AD, APP\textsuperscript{swen}, PS1\textsuperscript{M146V}, tauP301L; AD, Alzheimer's disease; APN/CD13, aminopeptidase N; APP, amyloid precursor protein; ATG3, autophagy-related protein 3; ATG5, autophagy-related protein 5; Aβ, amyloid beta peptide; BBB, blood-brain barrier; BCA, bicinchoninic acid assay; BCEC, brain capillary endothelial cells; BM, Barnes maze; BSA, bovine serum albumin; CAA, cerebral amyloid angiopathy; CSF, cerebrospinal fluid; DEA, deethylamine; FA, formic acid; HCl, hydrochloric acid; HFD, high-fat-high cholesterol diet; HFIP, hexafluoro-2-propanol; HPR1-T, hypoxanthine phosphoribosyltransferase 1; HSP27, heat shock protein 27; IB, immunoblotting; IF, immune-fluorescent; IR(s), insulin receptor(s); IRS-1, insulin receptor substrate-1; IR-β, insulin receptor beta; KO, knock out; LAMP-1, lysosomal-associated membrane protein 1; LC3B, microtubule-associated proteins 1A/1B light chain 3B; LRP-1, low-density lipoprotein receptor-related protein-1; MAPK, mitogen-activated protein kinase; mBCEC, murine brain capillary endothelial cells; mm, Mus musculus; NEFA, non-esterified fatty acids; NTG, non-targeting control; NTg, non-transgenic; GGT, oral glucose tolerance test; pBCEC, porcine brain capillary endothelial cells; PDGFR-β, platelet-derived growth factor receptor beta; PECAM/CD31, platelet endothelial cell adhesion molecule; PFA, paraformaldehyde; P3-K, phosphoinositide 3-kinase; PSMD4, 26S proteasome non-ATPase regulatory subunit 4; PVDF, polyvinylidene difluoride membrane; RT, room temperature; sFRP-1, soluble low-density lipoprotein receptor-related protein-1; ss, Sus scrofa

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1. Introduction

Insulin receptors (IRs) and their signaling apparatus are widely conserved in various regions of the brain (Kleinridders et al., 2014; Werther et al., 1987). Insulin is known to preserve cognition by conducting crucial neurotransphinic functions and by regulating energy metabolism in the brain. Insulin signaling pathways in the CNS, similar as in peripheral tissues, are mediated by the phosphoinositide 3-kinase (PI3-K) and mitogen-activated protein kinase (MAPK) cascades (Zeng et al., 2016). Mounting evidence suggests a crucial link between Alzheimer's disease (AD) and impaired brain insulin signaling (Liu et al., 2011b; Matsuoka et al., 2010; Mullins et al., 2017). First, epidemiological studies suggest an elevated risk of AD in diabetic patients (Talbot et al., 2012). Second, impaired brain insulin signaling was reported in two different AD mouse models, as well as impaired central and peripheral insulin signaling in young and aged APPswe PS1M146V, tauP301L (3XTg-AD) mice (Velasquez et al., 2017). Change in cerebral IR levels in human AD is still a matter of debate. For example, diminished IR and insulin like growth factor receptor (IGFR) gene levels were reported in AD post mortem brain tissue (Steen et al., 2005), and impaired response to insulin-induced activation of the insulin receptor substrate-1 (IRS-1) signaling pathway was detected in cortex tissue from post mortem AD brains (Talbot et al., 2012). In contrast, no such change in human IR protein levels were identified in AD post-mortem brain samples when compared to age matched healthy post-mortem brains, despite that IGFR and IR signaling was compromised in AD neurons suggesting that degenenrating neurons in AD may be resistant to IGF-1R/IR signaling (Moloney et al., 2010). Third, high-fat diet (HFD) feeding caused memory deficits in several transgenic mouse models of AD and these deficits coincided with peripheral and central insulin resistance (Knight et al., 2014; Pratchayasakul et al., 2011; Takeda et al., 2010). The 3XTg-AD mice demonstrate elevated brain soluble amyloid beta peptide (Aβ) levels and larger amyloid plaque burden upon long-term HFD feeding, which was previously shown to increase peripheral insulin resistance, and a single dose of intravenous (i.v.) insulin injection mitigated these effects (Vandal et al., 2014).

The blood-brain barrier (BBB) was assumed to be the main portal for insulin delivery into brain (Pardridge et al., 1985). The IR is expressed at the BBB and high affinity binding sites for insulin were identified on the luminal side of the BBB (Miller et al., 1994). Insulin uptake into brain appears to be facilitated by saturable, receptor-mediated endocytosis in BBB endothelial cells (Frank et al., 1986; Gray et al., 2017; Miller et al., 1994). With increasing age, the cerebral response to peripheral insulin declines in parallel with a reduced cerebrospinal fluid to serum ratio of insulin (Sartorius et al., 2015). Insulin signaling at the BBB is known to regulate cell proliferation and tight-junction integrity (Ito et al., 2017). However, the expression and regulation of IR-β, the major subunit of IR with intrinsic tyrosine kinase activity, and various insulin signaling components at the BBB in AD have not been thoroughly investigated.

Low-density lipoprotein receptor-related protein-1 (LRP-1) is expressed in BBB endothelial cells and represents the major multifunctional scavenging receptor responsible for brain to blood Aβ clearance, as well as Aβ clearance from plasma by the liver (Deane et al., 2004; Ramanathan et al., 2015; Shibata et al., 2000; Tamaki et al., 2006). The intracellular domain of LRP-1 binds to phosphatidylinositol-binding clathrin assembly protein to regulate endosomal transcytosis of Aβ at the BBB (Ramanathan et al., 2015; Zhao et al., 2015). LRP-1 expression levels are closely associated with lipid and Aβ homeostasis in brain and plasma. Low levels of LRP-1 were reported in AD patient's brain (Kang et al., 2000), cerebrovascularulature, and of soluble LRP-1 (sLRP-1) in plasma (Deane et al., 2004; Shibata et al., 2000). Functional knock-out (KO) of LRP-1 in murine brain capillary endothelial cells (mBCEC) from 5xFAD mice induced impaired Aβ clearance from the brain (Storch et al., 2016). Furthermore, Liu et al. reported impaired brain lipid metabolism and progressive age-dependent neurodegeneration in neuronal LRP-1 KO mice (Liu et al., 2010). In accordance with the above, an augmentation of sLRP-1 levels by i.v. infusion was shown to ameliorate brain Aβ pathology in APP + /sw mice (Sagare et al., 2007). Intriguingly, LRP-1 has been identified as a binding partner for IR and to affect insulin signaling upon dimerization with IR-β. LRP-1 knockout in mice reduced IR-β levels and activity, thereby provoking impaired insulin-mediated effects in the brain and in neurons (Fuentebalba et al., 2009; Liu et al., 2015).

Given the importance of LRP-1 for Aβ clearance (Storch et al., 2016) and IR-β for insulin responses and/or uptake at the BBB respectively, we aimed to uncover factors and mechanisms regulating LRP-1 and IR-β function in cerebrovascular insulin signaling in AD. For in vivo studies, we employed 3XTg-AD mice, carrying mutations for amyloid precursor protein (APPswe), presenilin 1 (PS1M146V) and tauP301L (Oddo et al., 2003). We emphasized on establishing the effects of HFD feeding on peripheral, cerebral and cerebrovascular LRP-1 and IR-β expression, comparing conditions of early Aβ pathology in 3XTg-AD with non-transgenic (NTg), as well as between male and female mice. Using a well-established in vitro primary porcine brain capillary endothelial cell (pBCEC) model, we examined more closely whether and how Aβ modulates insulin-mediated responses and LRP-1/IR-β homeostasis at the BBB. In addition, an intrinsic link between LRP-1 mediated IR-β regulation and insulin signaling was explored in pBCEC.

2. Material and methods

2.1. Animals and diet

Animal experiments were approved by The Austrian Federal Ministry of Science, Research and Economy (approval number 66.010/130-WF/V/3b/2015). Animals were maintained under standard housing conditions with controlled humidity, temperature, free access to water, and a 12 h light/dark cycle. Male and female 3XTg-AD (PS1M146V, APPswe and tauP301L) and NTg (C57/BL6) (Zenaro et al., 2015) mice were used for the study. Inbred 3XTg-AD mouse litters were genotyped for human APPswe and PS1M146V genes after extracting DNA from tail tips; PCR was performed using primers for human APPswe and PS1M146V provided by The Jackson Laboratories, and mice tested homozygous for both genes were selected for feeding studies (Supplementary Fig. 1). Mice were fed either high-fat/high-cholesterol diet (HFD) containing 60% kcal from fat (D12492, sniff® diets, Germany) or chow diet containing 12% kcal from fat, for 23 weeks starting from 12 weeks after weaning. Body weights were recorded weekly. Oral glucose tolerance test (OGTT; see2.2.) and Barnes Maze test (BM; see2.3.) were performed after 13 and 20 weeks of feeding, respectively. In female mice the stage of estrus cycle was assessed by vaginal smear analysis (Caligioni, 2009) on the day before sacrifice, which indicated that regardless of the feeding conditions both 3XTg-AD and NTg mice were in transition between metestrus (presence of cornified and nucleated epithelial cells) and anestrus cycle (presence of leukocytes). On day of sacrifice (age: 35 ± 2.7 weeks, referred to as 9-month-old), animals were fasted (6 h) and euthanized under CO2 flow. Blood was collected by cardiac puncture and plasma was prepared by centrifuging (2000 × g, 15 min). Cerebral microcapillaries were isolated as described under 2.5. Plasma and organs were stored at ~80°C for further analysis. Plasma insulin levels were determined using Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, USA). Plasma levels of non-esterified fatty acids (NEFA), cholesterol and triglycerides were determined using enzymatic kits provided by DiaSys Diagnostic Systems GmbH, according to the manufacturer's instructions.

2.2. Oral glucose tolerance tests

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).

2.3. Behavioral tests

Spatial learning and memory was assessed by using the BM test as previously described (Attar et al., 2013; Pitts, 2018). In brief, mice were placed on an elevated round wooden board (diameter: 90 cm) with 20 equally-spaced holes (diameter: 5 cm) at the edge. Mice were habituated to this maze (day 1) and then trained to find a target hole connected to a hidden black escape box (11 × 5 × 5 cm) beneath the maze for 3 days (2 trials/day). The latency to approach the first hole was used to identify any influence on locomotor or anxiety-like behavior, while the latency to find the target-hole was used to assess learning capabilities. The behavior of the animals during the trials was videotaped and tracked with VideoMot 2 software (TSE Systems, Bad Homburg, Germany).

2.4. Amyloid-β ELISA

Frozen brain cortical hemispheres from male and female 3XTg-AD mice were used to isolate diethylamine (DEA; Sigma-Aldrich) soluble and formic acid (FA; Sigma-Aldrich) soluble Aβ applying the method described by (Casali and Landreth, 2016). In brief, mechanically homogenized brain lysates were mixed with equal volumes of 0.4% DEA solvent (200 µl DEA, 1 ml of 5 M NaCl and 50 ml of ddH2O) and were centrifuged at 135,000 × g for 1 h at 4 °C. The supernatant was neutralized with 0.5 M Tris-HCl and stored at ~80 °C. The resulting pellet was sonicated on ice in 95% FA (~20 s with 15 mA amplitude) until dissolved, and centrifuged at 109,000 × g for 1 h at 4 °C. The supernatant was neutralized with FA neutralization buffer (1 M Tris, 0.5 M Na2HPO4 and 0.05% NaN3) and the samples were stored at ~80 °C. Levels of DEA and FA soluble Aβ1–40 and Aβ1–42 were determined using ELISA kits from ThermoFisher Scientific according to the manufacturer's indications. Quantification was achieved by generating a standard curve using a 4 parameter algorithm to obtain the best fit. Peptide concentrations were normalized to total brain protein content determined by BCA assay (pg/mg wet weight).

2.5. Isolation of murine brain microvessels

Brains were harvested and microvessels were isolated from 3 to 4 pooled brain hemispheres (Zandl-Lang et al., 2018). In brief, brain hemispheres devoid of meninges and olfactory bulbs were homogenized in MCDB131 medium (containing 2% FBS, 1% l-glutamine and 1% penicillin/streptomycin), subjected to disperse digestion (0.01 g/2 hemispheres, 1 h, 37 °C; Life Technologies), followed by a centrifugation step (6800 × g) using dextran solution (1:1 v/v, 1.0612 g/L; Alfa Aesar), and followed by brief collagenase/dispose (3.5 mg/20 ml, 1 min, 37 °C; Roche) digestion to detach the basement membrane from the endothelium, filtering the suspension through a nylon mesh (180 µm), and purification of microcapillary fragments after centrifugation in a Percoll (Sigma-Aldrich) density gradient from which mBCEC were collected from the interface. Resuspended cell pellets were strained through a nylon mesh (180 µm pore size) to purify microvessels. Homogenate containing microvessels was briefly incubated with collagenase/dispose (3.5 mg/20 ml) for 1 min at 37 °C to disrupt basement membranes, followed by purification of cerebrovascular fragments/BCEC using a biphase percoll gradient (20 ml of 1.03 g/ml percoll solution underlaid with 15 ml 1.07 g/ml percoll solution) and centrifuging at 1300 × g for 10 min at room temperature (RT). BCEC collected at the percoll biphase gradient interface were washed and cultured in collagen coated 75 cm² flasks supplemented with M199 medium (1% penicillin/streptomycin, 1 mM l-glutamine, 10% horse serum, 95% humidity, 5% CO2). After reaching 80–90% confluency, cells were trypsinized and seeded onto 6- or 12-well plates or chamber slides (Lab-Tek) for further experiments.

2.7. Preparation of insulin and Aβ peptides for in vitro experiments

Human recombinant insulin (Calbiochem®, Merck) was dissolved in 0.01 N hydrochloric acid (HCl) to obtain a 10 mM stock solution, stored at 4 °C, and used within 4 weeks according to manufacturer's instructions (Chiracall Maanavalan et al., 2014). Amyloid beta peptides (Aβ1–40 and Aβ1–42) were prepared according to manufacturer's instructions (rPeptide, Watkinsville, GA, USA). In brief, hexafluoro-2-propanol (HFIP) treated Aβ1–40 and Aβ1–42 films were dissolved in 1% ammonium hydroxide (Sigma-Aldrich) to obtain a concentration of 1 mg/ml. Then, solutions were briefly vortexed and sonicated in a water bath for 30 s until they were clear and free from aggregates. Aβ preparations were frozen in aliquots at ~80 °C. On day of use, fresh Aβ1–40 and Aβ1–42 aliquots were brought to room temperature and were immediately used. Aggregation profiles of Aβ1–40 and Aβ1–42 peptides are shown in the Supplementary Fig. 2. For all in vitro experiments in pBCEC, 240 nM (1 µg/ml) of Aβ1–40 and Aβ1–42 peptides were used.

2.8. SDS-PAGE and immune-blotting (IB)

Brain cortical tissue, liver, mBCEC, or pBCEC were lysed by brief sonication in protein lysis buffer (containing 50 mM Tris, 10 mM EDTA, 1% Triton X 100, phosphatase inhibitor (PhosSTOP™), protease inhibitor cocktail (Roche Diagnostics cobblestone™), and pH adjusted to 7.5). Protein quantification was performed using bicinchoninic acid assay (BCA, ThermoFisher Scientific). Samples were mixed (4:1) with 1× MOPS or MES (Life Technologies) buffer. Then, proteins were electrophoresed onto 0.45 µm polyvinylidene difluoride membranes (PVDF; GE Healthcare). Membranes were blocked with 5% non-fat dry milk peptide (Bio-Rad) for 1 h at room temperature (RT). Membranes were incubated with primary antibody overnight in 5% bovine serum albumin (BSA; Roche) at 4 °C, followed by HRP-conjugated secondary antibody incubation in 5% non-fat dry milk protein for 1 h at RT. Detection of immune-reactive bands was performed using Clarity Western ECL Substrate (Biorad) and a ChemiDoc imager (Bio-Rad). Band intensities were determined using ImageLab software (version 5.2.1, Bio-Rad). All primary and secondary antibodies used are listed in Supplementary Table 1.

2.9. Immuno-fluorescent staining (IF)

For immune-cytchemistry (Zandl-Lang et al., 2018), pBCEC were cultured on Lab-Tek chamber slides (Thermo Fisher Scientific, NY, USA). Cells were fixed with acetone and air dried. Mouse brain
Fig. 1. Dysregulated metabolic profile in 3XTg-AD and HFD-fed mice. (A) Animal body weights were monitored every week until the end of the 23-week feeding regimen. Data represent mean ± 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) (p < 0.001) and NTg HFD (F) vs 3XTg-AD HFD (F) (p < 0.001). (B) Oral glucose tolerance test (OGTT) was performed in mice fasted for 6 h. Data represent mean ± 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) (p < 0.05), 3XTg-AD chow (M) vs 3XTg-AD chow (F) (p < 0.05) and NTg HFD (M) vs 3XTg-AD HFD (M) (p < 0.05). (C) Daily food intake. After 23 weeks of feeding, animals were fasted, plasma was isolated and plasma parameters such as (D) insulin, (E) non-esterified fatty acids (NEFA), (F) triglycerides (TG), and (G) cholesterol were determined as described in material and methods. For C, D, E, F and G, statistical differences between the groups were evaluated using two-tailed, unpaired students t-test. Data represent mean ± SD; N = 7–9 mice/group, except for the 3XTg-AD male chow fed group, where N = 4 mice, *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); *p < 0.001), (3XTg-AD chow (M) vs 3XTg-AD chow (F); *p < 0.05), (NTg HFD (M) vs NTg HFD (F); *p < 0.001) and (3XTg-AD HFD (M) vs 3XTg-AD HFD (F); *p < 0.001).
immune-fluorescent (IF) stainings were carried out on 18 μm cryosections of cortex and fixed with 4% paraformaldehyde (PFA). Cells or sections were rinsed and blocked with donkey serum prior to primary antibody incubation for 1 h. Negative controls were incubated with the appropriate IgG fractions as isotope controls. After TBST wash, secondary antibodies were applied. DAPI was used as a nuclei counter stain. Sections were rinsed with TBST before mounting with Vectashield mounting medium (Vector Lab, Inc., Burlingame, CA, USA). To acquire and analyze computerized images of sections and cells, a Leica DM4000 B microscope (Leica Cambridge Ltd.) equipped with Leica DFC 320 Video camera (Leica Cambridge Ltd.) was used. Antibodies used for the IF stainings are listed in Supplementary table 2.

2.10. LRP-1 and IR-β silencing in pBCEC

Three targeting siRNAs for each target gene, i.e. porcine LRP-1 (5′-GGAGGAGCUGUGAACAUA-3′, 5′-ACAAGCUGUGCCCUUGGA-3′ and 5′-CCUGACUGGUGUGUCAAAA-3′) and porcine IR-β (5′-GCGUUGGGAAUUAUAUA-3′, 5′-GAAUUGGAGCAUGUAA-3′ and 5′-ACA CAGGCUUUGAUAGA-3′) were purchased from Microsynth. Non-targeting control (NTC) siRNAs were purchased from Dharmacon Research. Primary pBCEC at 70–90% confluence were transfected with DharmaFECT transfection reagent (Dharmacon) following manufacturer’s instructions and as described in (Kober et al., 2017). In brief, three siRNAs for each target gene (LRP-1 or IR-β) were pooled in nuclease free water to attain 5 μM stock solutions. For silencing experiments, cells were cultured in 6-well plates and incubated with pooled LRP-1/IR-β/NTC siRNAs (50 nm) along with DharmaFECT transfection reagent (10 μl/well) in serum free (SF) medium according to the manufacturer’s instructions. After 24 h of incubation, medium was replaced with M199, containing 5% horse serum for 24 h. Cells were harvested at indicated time points and silencing efficiency was determined using qRT-PCR (2.11.) and IB. For insulin treatments, after 48 h silencing, cells were serum starved for 12 h in SF medium prior to insulin stimulation.

2.11. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total cellular RNA was isolated using TriReagent RT (MRC, USA). Complementary DNA was prepared using High Capacity Reverse Transcriptase Kit (Life Technologies) following the manufacturer’s guidelines. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix (Biorad) on a CFX96 Real time system (Biorad) following the manufacturer’s guidelines. Quantitative real-time PCR (qRT-PCR) was performed using Transcriptase Kit (Life Technologies) following the manufacturer’s instructions. Total cellular RNA was isolated using TriReagent RT (MRC, USA). Complementary DNA was prepared using High Capacity Reverse Transcriptase Kit (Life Technologies) following the manufacturer’s guidelines. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix (Biorad) on a CFX96 Real time system (Biorad). Relative gene expression was quantified using the 2−ΔΔCt method after normalizing the target gene expression with reference gene hypoxanthine phosphoribosyltransferase 1 (HPRT-1) (Kober et al., 2017; Zandl-Lang et al., 2018). Primer pairs used are listed in the Supplementary Table 3.

2.12. Quantification of LC3B dot coverage of cerebrovascular endothelial cells

Image data was acquired using the method previously described by Yang and colleagues (Yang et al., 2003). In brief, from each double immune-fluorescent image of 18 μm mouse brain cryosections stained for microtubule-associated protein 1A/1B light chain 3B (LC3B) and platelet endothelial cell adhesion molecule (PECAM/CD31), three 1 mm² fields were randomly chosen and LC3B dots on CD31 coverage area were counted. Image acquisition and digital image quantification were performed using NIH Image J software.

2.13. Statistical analysis

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 statistically significant. All data are presented as means ± SD, N referring to the number of mice in each group; n referring to the number of independent in vitro experiments with different pBCEC isolations performed. For BM test, statistical data analysis was performed on 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 mean ± SEM, N referring to the number of mice in each group.

3. Results

3.1. Plasma insulin and lipid profiles in chow vs HFD fed male and female 3XTg-AD and NTg mice

Male and female 3XTg-AD and NTg mice were fed with standard chow or HFD for 23 weeks. OGTT was performed after week thirteen, to assess the impact of HFD on peripheral glucose tolerance and insulin sensitivity. As expected, HFD feeding for 23 weeks induced significant body weight gain in both genders and genotypes (Fig. 1A), which was accompanied by decreased glucose tolerance (OGTT; Fig. 1B). ANOVA indicated that HFD feeding significantly enhanced body weight (BW) gain in NTg mice (male: F20,377 = 20.13, p < 0.0001 and female: F20,355 = 1.78, p < 0.0221) and in 3XTg-AD mice (male: F20,245 = 3.15, p < 0.0001 and female: F20,261 = 3.92, p < 0.0001) vs chow fed mice. Moreover, female 3XTg-AD mice showed a higher food intake resulting in higher BW gain (87 ± 20.1% vs chow fed 3XTg-AD mice; F20,261 = 3.92, p < 0.0001) than female NTg mice (39 ± 7.7% vs NTg chow fed mice; F20,305 = 1.77, p < 0.022). Interestingly, food intake in HFD fed male 3XTg-AD mice and NTg female mice was significantly lower (p < 0.001) compared to HFD fed counterparts (Fig. 1C). In correspondence to BW gain, HFD feeding impaired glucose tolerance in both NTg (male: F5,122 = 6.87, p < 0.0001 and female: F5,108 = 6.14, p < 0.0001) and 3XTg-AD (male: F5,160 = 5.02, p < 0.0001 and female: F5,106 = 3.15, p < 0.0001) mice vs chow fed mice. Interaction between sexes was observed in NTg and 3XTg-AD mice. HFD fed NTg female mice showed better glucose tolerance when compared to HFD fed NTg male mice (F5,108 = 2.62, p < 0.0282), corresponding to food intake and body weight gain. However, chow fed 3XTg-AD female mice showed better glucose tolerance when compared to male 3XTg-AD mice (F5,72 = 4.36, p < 0.0016). Interaction between ‘transgene’ (i.e. genetic differences) was observed in chow and HFD fed, NTg and 3XTg-AD male mice, NTg chow (M) vs 3XTg-AD chow (M) (F5,78 = 3.59, p < 0.0057) and NTg HFD (M) vs 3XTg-AD HFD (M) (F5,102 = 4.05, p < 0.0021). Low but not significantly different (p = 0.051) fasting plasma insulin levels (Fig. 1D) were detected in male 3XTg-AD vs NTg mice. Female NTg and 3XTg-AD mice showed significantly lower fasting plasma insulin levels when compared to their male NTg and 3XTg-AD counterparts. In NTg mice, HFD feeding significantly elevated plasma insulin levels by 326 ± 181.2% in male and 339 ± 205.6% in female NTg mice vs male 3XTg-AD (M) (F5,106 = 2.62, p < 0.0282) and female 3XTg-AD (F5,72 = 4.36, p < 0.0016) mice vs chow fed mice. Interaction between sexes was observed in NTg and 3XTg-AD mice. HFD fed NTg female mice showed better glucose tolerance when compared to HFD fed NTg male mice (F5,108 = 2.62, p < 0.0282), corresponding to food intake and body weight gain. However, chow fed 3XTg-AD female mice showed better glucose tolerance when compared to male 3XTg-AD mice (F5,72 = 4.36, p < 0.0016). Interaction between ‘transgene’ (i.e. genetic differences) was observed in chow and HFD fed, NTg and 3XTg-AD male mice, NTg chow (M) vs 3XTg-AD chow (M) (F5,78 = 3.59, p < 0.0057) and NTg HFD (M) vs 3XTg-AD HFD (M) (F5,102 = 4.05, p < 0.0021). Low but not significantly different (p = 0.051) fasting plasma insulin levels (Fig. 1D) were detected in male 3XTg-AD vs NTg mice. Female NTg and 3XTg-AD mice showed significantly lower fasting plasma insulin levels when compared to their male NTg and 3XTg-AD counterparts. In NTg mice, HFD feeding significantly elevated plasma insulin levels by 326 ± 181.2% in male and 114 ± 62.2% in female mice. Interestingly, no significant increase in insulin levels was observed in HFD fed 3XTg-AD mice (Fig. 1D). Significantly elevated NEFA (65 ± 9.2%), triglycerides (30 ± 6.5%) and cholesterol (62 ± 21.5%) levels were detected in female 3XTg-AD as compared to female NTg mice (Fig. 1E-G). While elevated plasma NEFA levels and triglycerides (3XTg-AD HFD (M) mice) were detected in both male and female 3XTg-AD mice independent of HFD feeding (Fig. 1E), no differences in plasma triglycerides and cholesterol were observed in male mice of either genotype (Fig. 1F&G).
3.2. Impact of gender and diet on cerebral Aβ burden and memory in 3XTg-AD mice

Since HFD feeding is known to aggravate brain Aβ pathology in older (15 months) female 3XTg-AD mice (Vandal et al., 2014), by using ELISA we quantified soluble (i.e. DEA extracted oligomeric and pre-fibrillary Aβ) and insoluble (i.e. FA extracted fibrillary Aβ aggregates) Aβ1–40 and Aβ1–42 in brain homogenates of our 9-month-old male and female, chow- and HFD-fed 3XTg-AD mice. Aβ1–40 and Aβ1–42 was not detectable in NTg mouse brain lysates (not shown). Overall, female 3XTg-AD mice revealed by 2.7 ± 0.83 and 5.2 ± 0.12-fold higher soluble and 4.8 ± 0.33 and 4.1 ± 0.14-fold higher insoluble Aβ1–40 and Aβ1–42 levels when compared to male mice (Fig. 2A&B). When on HFD, soluble Aβ1–40 levels were significantly higher in female HFD-fed 3XTg-AD mice (57 ± 10.0%) vs chow fed 3XTg-AD mice, but below detection limit in male mice. Male and female HFD-fed 3XTg-AD mice exhibited significantly higher soluble Aβ1–42 levels (males: 62 ± 9.1% and females: 91 ± 7.2%) vs chow fed 3XTg-AD mice (Fig. 2A). However, no significant changes were observed in insoluble Aβ1–40 and Aβ1–42 levels (Fig. 2B). In addition, Aβ targeted IF staining (using 6E10 antibody) of hippocampal cryosections revealed staining of Aβ plaques in brains of both chow and HFD fed male and female 9-month-old 3XTg-AD mice, whereas only faint staining was observed in NTg mice (Fig. 2C).

Since HFD was further reported to impair memory in old, female 3XTg-AD mice (Knight et al., 2014; Vandal et al., 2014), we also tested whether HFD feeding in 9-month-old male and female 3XTg-AD and NTg mice has effects on memory and anxiety behavior. Interestingly, BM analysis revealed significant behavioral changes reflecting anxiety and memory in female, but not in male 3XTg-AD mice. The latency to

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Fig. 2. Effect of HFD feeding, gender, and 3XTg-AD transgene on cerebral Aβ burden and memory. Soluble and insoluble Aβ was extracted from the cortices of 3XTg-AD mice. (A, B) Levels of diethylamine soluble (‘soluble’) and formic acid soluble (i.e. ‘insoluble’) human Aβ1–40 and Aβ1–42 in total brain parenchyma of chow and HFD-fed 3XTg-AD mice, quantified by ELISA (N = 4 mice per group). Data represent means ± SD. Statistical significance was determined using two-tailed, unpaired students t-test (p < 0.05 and **p < 0.01). C) Representative immune-fluorescent staining of Aβ burden in the hippocampus of chow and HFD fed 3XTg-AD and NTg, male and female mice. DAPI staining in blue, 6E10 staining in red. (D–G) At the end of 20 weeks during the course of HFD feeding animals were tested for anxiety and memory using Barnes Maze (BM). (D, F) Target hole latency (memory), (E, G) First hole latency (anxiety). N = 8 mice/group, except for 3XTg-AD male chow fed, where N = 4 mice/group. Data represent mean ± 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. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)
find the target hole revealed that the learning performance of female mice over multiple trials correlated with both the genotype and the diet (Fig. 2D): female 3XTg-AD mice on chow or HFD feeding showed impaired target hole latency ($F_{1,27} = 20.161; p < 0.001$ and $F_{1,27} = 6.308; p = 0.018$ vs respective chow and HFD fed NTg controls) (Fig. 2D); first-hole latency time, a parameter to determine anxiety, worsened upon HFD feeding in female 3XTg-AD mice ($F_{1,27} = 8.166; p = 0.008$ vs NTg controls) (Fig. 2E). However, no such differences in anxiety and/or memory were observed in male 3XTg-AD mice of both feeding regimens (Fig. 2F&G).

3.3. The 3XTg-AD transgene and HFD feeding affect liver LRP-1 and IR-β expression

Since insulin mediates LRP-1 regulation and plays a crucial role also in peripheral Aβ clearance (Tamaki et al., 2006; Tamaki et al., 2007), we first explored if IR-β and LRP-1 expression are modulated in peripheral tissue. Therefore, we subjected liver lysates of 3XTg-AD and NTg mice to IB and determined LRP-1, IR-β, and pAKT/AKT protein levels. Liver IR-β levels were moderately but significantly down-regulated by 26 ± 4.5% in male and 25 ± 4.0% in female chow fed 3XTg-AD mice as compared to NTg mice (Fig. 3A&B). Similarly, feeding NTg mice a HFD led to decreased IR-β levels by 22 ± 1.3% when compared to chow fed male but not female mice (Fig. 3A&B). Lower hepatic IR-β levels in 3XTg-AD mice maintained on either feeding regimens coincided with lower circulating insulin levels, which is indicative of hepatic insulin resistance. Interestingly however, chow fed 3XTg-AD mice did not show altered hepatic LRP-1 levels (Fig. 2C), but HFD feeding significantly decreased hepatic LRP-1 levels by 54 ± 21.0% and 49 ± 18.9% in both NTg and 3XTg-AD males compared to the corresponding chow diet controls (Fig. 3A&C). The pAKT levels in liver lysates were significantly elevated by 105 ± 22.5% in males and 106 ± 32.2% in females 3XTg-AD chow fed mice compared to NTg mice (Fig. 3A&D). However, HFD feeding significantly decreased hepatic pAKT levels in male and female 3XTg-AD mice (Fig. 3A&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. Together, these results indicate that 3XTg-AD mice exhibited impaired hepatic IR-β levels and hyper-activated downstream AKT levels; HFD feeding exerted a modest effect on hepatic IR-β levels in these mice. In contrast, HFD feeding clearly induced downregulation of LRP-1 levels in livers of both genotypes.

3.4. Cerebral IR-β/LRP-1 homeostasis and insulin signaling is impaired in 3XTg-AD mice

We next analyzed the effect of HFD feeding on IR-β/LRP-1 homeostasis and insulin signaling in the brain (Fig. 4A&B). 3XTg-AD mice exhibited marked reductions in brain IR-β protein levels by 49 ± 13.7% in males and 35 ± 6.6% in females compared to NTg mice (Fig. 4A&C). HFD feeding further decreased cerebral IR-β levels by 30 ± 5.4% in 3XTg-AD female mice but not in male mice compared to their Chow fed counterparts (Fig. 4C). The general pattern of lower IR-β levels in brain lysates of 3XTg-AD vs NTg mice was also evident (Fig. 4C). Moreover, a reduction in pIRS-1 (S1101) levels was detected in brain lysates of male 3XTg-AD mice by 37 ± 7.5% and female mice by 47 ± 25.8% compared to Chow fed NTg mice (Fig. 4E). However, HFD feeding did not lead to decreased IRS-1 phosphorylation in brain, irrespective of genotype or gender; on the contrary, female NTg mice on HFD exhibited increased IRS-1 levels, $p < 0.001$ vs Chow fed NTg mice (Fig. 4E). Furthermore, Chow fed 3XTg-AD mice exhibited increased phosphorylation of cerebral AKT and ERK as compared to NTg mice of same gender (Fig. 4F&G). HFD feeding decreased cerebral pAKT but not pERK levels in 3XTg-AD mice (Fig. 4F&G). In contrast, HFD feeding of NTg mice did not affect cerebral pAKT or pERK levels in male mice but led to increased pERK levels in female mice (Fig. 4F&G). Interestingly, a significant reduction in LRP-1 protein levels by 57 ± 34.4% in the brain lysates of male and 46 ± 21.9% in female

Fig. 3. Effect of HFD feeding and 3XTg-AD transgene on liver IR-β/LRP-1 axis. At the end of the feeding regimen (23 weeks), liver lysates were prepared from mice of both genotypes and genders, and were subjected to immune-blotting using polyclonal antibodies. (A) Representative blots for IR-β, LRP-1, pAKT(s473), total AKT and GAPDH. (B) Quantitative, densitometric analysis of (D) IR-β, LRP-1, pAKT(s473), total AKT and GAPDH. Data represent mean ± SD. Statistically significant differences between the groups were calculated using two-tailed, unpaired students t-test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. **
3XTg-AD mice was observed as compared to chow fed NTg animals (Fig. 4D), which was paralleled with reduced brain IR-β levels. HFD feeding decreased LRP-1 expression in brains of NTg mice of both genders (by 46 ± 6.9% & 28 ± 1.2% in male and female mice, respectively) as compared to chow fed NTg mice, but not in that of 3XTg-AD mice (Fig. 4B).

3.5. IR-β/LRP-1 homeostasis is dysregulated in cerebrovascular endothelial cells in 3XTg-AD mice

Since insulin mediates LRP-1 trafficking in cerebrovascular endothelial cells (Swaminathan, 2017), and LRP-1 is known to regulate Aβ clearance from the brain via the cerebrovasculature (Storck et al., 2016), we next investigated whether LRP-1 as well as IR-β levels were altered at the level of the BBB in 3XTg-AD mice (Fig. 4B).

Fig. 4. Effects of HFD feeding and 3XTg-AD transgene on brain IR-β/LRP-1 axis and downstream insulin signaling. Immunoblot analyses of brain lysates were performed and changes in IR-β/LRP-1 levels and insulin signaling events were analyzed using specific antibodies. A, B: Representative immune-blots 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 analysis of specific bands for (C) IR-β (N = 5) and (D) LRP-1 (N = 5) as normalized to β-actin. Quantitative analysis of (E) pIRS-1(S1101) (N = 5), (F) pAKT(s473) (N = 5) and (G) pERK (N = 5), where band intensities were normalized to respective total protein band intensities. Data represent mean ± 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.
Fig. 5. Perturbed IR-β/LRP-1 axis at the BBB in 3XTg-AD mice. At the end of the HFD feeding period (23 weeks), fasted animals were sacrificed. Cerebromicrovasculature was isolated as described in Material and methods. (A) IR-β and LRP-1 presence in mBCEC. Isolated cerebral microvessels were immune-fluorescently stained for (a) CD31 (red) and IR-β (green); (b) CD31 (red) and LRP-1 (green); (c) IR-β (magenta) and LRP-1 (green). DAPI (nuclear stain) staining in blue. Co-localization of IR-β and LRP-1, shown in white, represented by yellow arrows. (B-D) Murine BCEC protein lysates of male and female 3XTg-AD and NTg mice of both feeding regimens were subjected to immunoblot analyses. (B) Representative blots for IR-β, LRP-1, pAKT, total AKT and GAPDH levels. Densitometric analysis of specific bands for (C) IR-β (N = 4) and (D) LRP-1 (N = 4) as normalized to GAPDH as loading control. Data represent mean ± 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. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)
when compared to chow fed NTg mice (Fig. 5B&D). As opposed to brain LRP-1 levels, the HFD mediated effects on LRP-1 levels in microvessels purified from male or female 3XTg-AD mice were not very pronounced when compared to chow fed 3XTg-AD mice (Fig. 5C&D).

3.6. Elevated brain and cerebrovascular autophagy in 3XTg-AD mice

Since 3XTg-AD mice showed elevated brain Aβ peptide levels, we were interested to know the status of autophagy and its correlation with impaired LRP-1 and IR-β levels at the cerebrovascular level. Double immune-fluorescent images of CD31 and LC3B staining revealed that LC3B dot coverage in cerebrovascular endothelial cells was significantly higher in both male and female 3XTg-AD mice (190 ± 36% and 266 ± 43%) vs respective chow fed NTg mice (Fig. 6A–C). HFD feeding further elevated LC3B dot coverage in cerebrovascular endothelial cells identified in cortical crysections obtained from 3XTg-AD (31 ± 8% and 28 ± 6% from male and female HFD fed 3XTg-AD mice vs chow fed 3XTg-AD mice) but not from NTg mice (Fig. 6A–C). In whole brain lysates, 3XTg-AD mice (independent of gender) showed elevated LC3B-II levels demonstrating active autophagy (92 ± 16% and 81 ± 14%) in male and female 3XTg-AD mice vs respective NTg mice; Fig. 6D). Feeding the HFD elevated brain LC3B-II levels significantly in both genotypes and genders (73 ± 15% and 62 ± 7% in male and female HFD fed NTg mice vs respective chow fed NTg mice; 167 ± 37% and 212 ± 41% in male and female HFD fed 3XTg-AD mice vs respective chow fed NTg mice) (Fig. 6D&E). Elevated LC3B levels in brain cortex and cerebral vessels in 3XTg-AD mice correlated with changes in brain Aβ burden (Fig. 2A&B) and changes in LRP-1 and IR-β receptor levels (Fig. 5B–D).

3.7. Aβ treatment disrupts insulin induced LRP-1 expression, IR-β levels, and insulin signaling in an in vitro model of the BBB

Since cerebrovascular LRP-1 and IR-β levels were downregulated in 3XTg-AD mice, effects of Aβ peptides on BBB endothelial LRP-1 and IR-β receptors were explored. Incubation of cultured pBCEC with Aβ₁₋₄₀ or
Aβ1–42 significantly downregulated insulin induced upregulation in LRP-1 levels by 76 ± 28.3% and 75 ± 32.6%, respectively, as compared to insulin treated pBCEC, which is evident from immune-blots (Fig. 7A). Aβ1–40 or Aβ1–42 treatment alone induced significant downregulation of LRP-1 levels by 44 ± 14.8 and 61 ± 16.4, respectively. In parallel, a significant downregulation in IR-β protein levels by 43 ± 13.3% and 50 ± 11.6%, respectively, when compared to vehicle treatment was detected. Enhanced LC3B-I levels were observed upon Aβ incubation, as indicated in immune-blots and quantifications (Fig. 7A&B), indicating autophagy induction. In line with the IB results, double IF stainings for IR-β and LRP-1 revealed reduced receptor expression and co-localization upon Aβ1–40 treatment of pBCEC (Fig. 7C). Since both Aβ isoforms clearly affected IR-β levels in pBCEC, we further examined their effects on insulin-mediated signaling by investigating pAKT and pERK protein levels. As expected, we found that insulin treatment of pBCEC increased AKT(S473) phosphorylation by...
461 ± 35.2% vs vehicle treatment (Fig. 7D&E). In sharp contrast, Aβ1–40 or Aβ1–42 treatment of pBCEC in the presence of insulin markedly reduced AKT phosphorylation by 67 ± 8.5% and 85 ± 21.8%, respectively, as compared to insulin treated pBCEC (Fig. 7D&E). Incubation of pBCEC with insulin also increased ERK (42/44) phosphorylation by 114.5 ± 18.4% as compared to vehicle treatment (Fig. 7D&F). However, incubation of pBCEC with either Aβ1–40 or Aβ1–42 alone (in the absence of insulin) led to similarly increased ERK phosphorylation by 121 ± 21.1% and 151 ± 56.6%, respectively, as compared to vehicle treated pBCEC (Fig. 7D&F).

3.8. Aβ induces autophagy-lysosomal degradation of LRP-1 and IR-β in pBCEC

Since cell surface receptor recycling and misfolded protein degradation is controlled by cell autophagy-lysosomal degradation pathway in cooperation with ubiquitin-proteasomal pathway (Kocaturk and Gozuacik, 2018; Lilienbaum, 2013), we next investigated whether these pathways are modulated by Aβ peptides in pBCEC. Lysosomal induction was observed in pBCEC upon Aβ1–40 treatment, as indicated by enhanced lysosomal-associated membrane protein 1 (LAMP-1) levels.
in Aβ1–40 treated cells (Fig. 8A,a–d). LRP-1 and IR-β trafficking to lysosomes (LAMP-1, red) was observed, as indicated by co-localization of LAMP-1 (red) with IR-β (green) or LRP-1 (green) in Aβ1–40 treated cells (Fig. 8A,c&d). We next studied effects of Aβ treatment on autophagy-lysosomal induction by using specific markers for lysosomal induction (LAMP-1) and autophagy such as LC3B, autophagy-related protein 3 (ATG3) and autophagy-related protein 5 (ATG5). A significant upregulation of LAMP-1 levels by 102 ± 10.4% as compared to vehicle control was identified in Aβ treated cells (Fig. 8A,B&D), which correlated with increased ATG5 (by 87 ± 5.6%) and ATG3 (by 56 ± 15.1%) levels, as compared to vehicle treatment, and significantly reduced LRP-1 and IR-β protein levels, by 59 ± 18.6% and 58 ± 10.6%, respectively, vs vehicle treated pBCEC cells (Fig. 8A,B&D). Treatment with lysosomal inhibitor (chloroquine) rescued Aβ mediated lysosomal degradation of LRP-1 and IR-β proteins. Proteasome inhibition with MG132 partially rescued LRP-1 and IR-β proteins. Both occurred predominantly by blocking Aβ induced autophagy-lysosomal induction, as depicted by significantly reduced LAMP1 levels upon treatment with Aβ together with chloroquine (by 75 ± 31.5% vs Aβ1–40 treatment) or MG132 (by 57 ± 19.0% vs Aβ1–40 treatment) (Fig. 8A,B&D, B,e–l). Chloroquine treatment significantly impaired autophagy-lysosomal degradation in pBCEC, represented by LC3B accumulation as indicated by IB (Fig. 8A,B&D) and IF staining (Fig. 8B,m–p), reduced ATG3 and ATG5 protein levels (Fig. 8A,C&D). In addition, chloroquine treatment significantly reduced proteasomal regulatory proteins, 26S proteasome non-ATPase regulatory subunit 4 (PSMD4), and heat shock protein 27 (HSP27). These findings together indicate that by inhibiting Aβ induced autophagy-lysosomal activity, LRP-1 and IR-β degradation is rescued in pBCEC.

3.9. LRP-1 is important for post-translational regulation of IR-β and insulin signaling in pBCEC

Having established the effects of Aβ treatment on IR-β and LRP-1 expression and their interaction in pBCEC, we next assessed a possible crosstalk between LRP-1 and IR-β that might cause these effects. Therefore, pBCEC were transfected with either LRP-1, IR-β, or mock (non-targeting control, NTC) silencing (si)RNAs. Gene silencing efficiency was confirmed by diminished mRNA expression levels of LRP-1 (47 ± 7.4%) and IR-β (53 ± 12.7%) using qRT-PCR (Fig. 9A&B). The amount of IR-β transcript remained unaltered when LRP-1 was silenced and vice versa (Fig. 9A&B), indicating that no crosstalk between IR-β and LRP-1 occurred at the mRNA level. However, LRP-1 silencing markedly reduced the protein levels of IR-β by 60 ± 9.2% vs NTC siRNA treated pBCEC, independently of insulin treatment of pBCEC (Fig. 9A&F). Similarly, insulin-mediated phosphorylation of IR-β (Tyr1131) and AKT (S473) was hampered by 80 ± 7.2% and 90 ± 7.5%, respectively, upon LRP-1 silencing in pBCEC (Fig. 9F&G). In contrast, silencing of IR-β exhibited no effect on LRP-1 protein levels, while it decreased insulin-mediated phosphorylation of IR-β (Tyr1131).
In our studies, 3XTg-AD mice showed elevated plasma lipid levels, in- 

vivo Aβ production in the 3XTg-AD model and when added 

bromicrovascular insulin signaling. Together, our results suggest that 

further understand dysregulated insulin signaling in early AD pa-

tiology, with a specific focus on LRP-1 and IR-β function in cere-

bral capillary endothelial cell (pBCEC) BBB model to 

regulate neuronal IR-β expression and modulate insulin signaling in the 

brain (Fuententhalba et al., 2009; Liu et al., 2015). Less is known about the 

interplay and regulation of IR-β and LRP-1 at the BBB and how it is 

influenced in/by AD. This study employed the 3XTg-AD mouse model 

and AKT (S473) in pBCEC by 88 ± 4.2% and 91 ± 5.9% vs insulin 

treated control cells (Fig. 9F&G), independent of changes in LRP-1. In 

summary, these results suggest that LRP-1 is associated with IR-β re-

ceptor expression in BBB endothelial cells and loss in LRP-1 leads to 

impaired IR-β levels, which in turn affects insulin signaling in pBCEC.

4. Discussion

Aberrant brain insulin signaling is a known hallmark of AD in-

dividuals (Bedse et al., 2015; Frolich et al., 1998; Frolich et al., 1999; 

Matsuzaki et al., 2010), and LRP-1 has been demonstrated recently to 

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phosphorylation of upstream mediators like IR and receptor associated proteins. Such negative feed-back mechanism however does not fully explain the present findings, particularly given that ERK signaling in 3XTg-AD mice and AKT signaling in HFD-fed mice were affected as predicted, i.e. decreased pAKT and elevated ERK levels (Fig. 4F&G). Even though ERK is activated in response to insulin and has a positive effect on cell growth, it also participates in insulin signaling malfunc-
tion and its inhibition can prevent TNFα-induced insulin resistance (Engelman et al., 2000; Ghasemi et al., 2014; Subramaniam and Unsicker, 2010). Similarly, IR-β silencing in pBCEC resulted in decreased AKT signaling. (Fig. 9C&G). However, increased AKT activity along with decreased IR-β levels (at the BBB) in 3XTg-AD mice is in good agreement with another working hypothesis for how diabetes modifies AD risk suggesting that reduced (central) insulin signaling in diabetes results in GSK3B activation, tau hyperphosphorylation, and NFT formation (Jolivalt et al., 2008; Steen et al., 2005; Yang et al., 2013).

Diminished IR-β levels in brain and at the BBB coincided with changes in LRP-1 levels in 3XTg-AD mice (Fig. 5B–C). Insulin was shown to elevate neuronal LRP-1 levels (Liu et al., 2015) and is known to promote surface mobilization of LRP-1, thus improving LRP-1 mediated Aβ clearance in hepatocytes (Tamaki et al., 2007) and also at the BBB (Swaminathan, 2017). As previously reported, LRP-1 has the ability to mediate insulin signaling by dimerizing with IR-β; knocking out LRP-1 adversely affects insulin-mediated responses in neuronal cells (Fuentealba et al., 2009; Liu et al., 2015). Moreover, reduced brain and cerebrovascular LRP-1 levels were reported in human subjects with AD (Deane et al., 2004; Kang et al., 2006; Shibata et al., 2000).

Our results of decreased brain LRP-1 (Fig. 4D) and IR-β levels (Fig. 4C) as well as impaired IKS-1 activation (Fig. 4E) in 3XTg-AD mice indicate that this mouse model resembles a central IR-β/LRP-1 defective phenotype (Liu et al., 2015). Our study revealed that HFD feeding further aggravates LRP-1 dysregulation in peripheral (liver; Fig. 4C) and central (brain; Fig. 4D) compartments, however without having significant effects on cerebrovascular LRP-1 levels. Hepatic LRP-1 was reported to regulate insulin signaling and its deletion causes insulin resistance thereby attributing to increased body weight gain, dyslipidemia, glucose intolerance, and low surface IR expression (Au et al., 2017). Since LRP-1 regulates energy homeostasis via leptin signaling (Liu et al., 2011a), impaired cerebral and hepatic LRP-1 levels in 3XTg-AD and HFD fed mice may contribute to dysregulated brain and peripheral energy homeostasis. Surprisingly, HFD-induced LRP-1 dysregulation did not affect IR-β levels in brain and liver, indicating that metabolic changes upon HFD feeding independently regulate LRP-1 and IR-β homeostasis.

Under insulin resistant or deprived states as manifest in HFD fed 3XTg-AD mice, insulin may not efficiently regulate LRP-1 expression, thereby contributing to impaired central and peripheral Aβ clearance. Regarding the question on the direction of causation between diabetes and AD, our data support a ‘reverse causation’ with AD driving diabetes, consistent with the development of a vicious cycle between AD and diabetes as proposed by Vandal et al. (2014) based on their findings of glucose intolerance in 3XTg-AD mice resulting from pancreatic human Aβ accumulation and which was rescued by insulin. We here observed a potentially additive vicious cycle in the brain of 3XTg-AD mice, where Aβ production in the brain parenchyma acts on BCEC to reduce both LRP-1 and IR-β, which consequently hampers insulin mediated effects on LRP-1 and Aβ clearance to the periphery. HFD feeding may further worsen central and peripheral Aβ clearance by diminishing pancreatic insulin production and by downregulating LRP-1 levels in central (brain) and peripheral (liver) compartments.

Although the 3XTg-AD mouse model develops cerebral/neuronal Aβ deposition very early (Oddo et al., 2003) as confirmed in the present study by detection of soluble and insoluble brain Aβ levels, 6E10 IF staining, and congo red staining in male and female mice (Fig. 2A–C, Supplement Fig. 5A&B), incidence of cerebral amyloid angiopathy (CAA), predominantly represented by cerebrovascular insoluble Aβ fibril accumulation was not evident in 9-month-old 3XTg-AD female or male mice (Supplement Fig. 5E–H). However, the cerebrovasculature is known to interact and transport soluble Aβ, predominantly via endothelial LRP-1, which is known to regulate about 50% of total brain Aβ clearance in a mouse model of AD (Storck et al., 2016). It is thus conceivable that increased Aβ challenge does occur at the level of the BBB under conditions of early detectable Aβ deposition in brain parenchyma only, as in the present study.

Recent evidences suggest that Aβ peptides induce autophagy in cerebrovascular endothelial cells in mouse (Hayashi et al., 2009) and humans (Rajadas et al., 2013). We observed a profound increase in LC3B-II levels in 3XTg-AD brain lysates indicating active autophagy in 3XTg-AD mice and HFD fed NTg mice (Fig. 6D&G). LC3B and CD31 immune-fluorescent quantification in cerebral vessels revealed a higher number of LC3B dots in male and female 3XTg-AD as compared to NTg mice (Fig. 6A–C) which was further enhanced by HFD feeding in 3XTg-AD but not in NTg mice. Even though HFD feeding elevated LC3B levels in NTg mouse brain, LC3B coverage on cerebral vessels was unchanged compared to chow fed NTg mice (Fig. 6A–E). It is thus conceivable that in 3XTg-AD mice increased Aβ levels correlate with enhanced cerebrovascular LC3B levels and reduced LRP-1 and IR-β receptor levels.

To understand the molecular basis of Aβ driven LRP-1 and IR-β receptor downregulation at the BBB, we investigated the pathways regulating protein turnover and degradation and their impact on Aβ mediated degradation of LRP-1 and IR-β, particularly autophagy-lysosomal proteolysis, also termed autophagy (Fecto et al., 2014). The latter predominantly regulates surface receptor recycling and is next to the ubiquitin-proteasomal pathway primarily involved in degradation of misfolded proteins. We chose to pursue mechanism of Aβ induced autophagy-lysosomal dysregulation using Aβ40 as since this is the most abundant soluble amyloid peptide found in biological fluids and in cerebrovascular amyloid plaques (Hayashi et al., 2009; Zlokovic et al., 1996). Using cultured pBCEC, we identified that Aβ has the ability to induce lysosomes, represented by enhanced cellular LAMP-1 levels and autophagy, represented by elevated levels of LC3B and ATGs (Fig. 8B–D). Using chloroquine (lysosomal inhibitor), we were able to rescue Aβ mediated autophagy-lysosomal degradation of LRP-1 and IR-β proteins in pBCEC. Moreover, chloroquine treatment blunted Aβ induced LAMP-1 upregulation as well as autophagy as indicated by reduced ATG levels, and enhanced LC3B accumulation, suggesting that autophagy-lysosomal degradation is impaired (Fig. 8D). MG132 treatment partially rescued LRP-1 and IR-β protein levels, since it affected Aβ induced LAMP-1 activity without affecting autophagy, in fact MG132 induced autophagic flux as indicated by enhanced LC3B-II levels, cytoplasmic inclusions and marginal upregulation of ATGs, which are responsible for autophagophore formation and maturation (Mizushima et al., 2011) (Fig. 8A–D). These findings further explain the earlier reported phenomenon of elevated LC3 and LAMP-1 levels observed in brains of 3XTg-AD mice (Villamil-Ortiz and Cardona-Gomez, 2015).

While it is well established that neuronal LR1 plays a critical role in Aβ clearance (Kanekiyo et al., 2013), it also influences glucose metabolism and neuronal insulin signaling by regulating IR-β expression (Fuentealba et al., 2009; Liu et al., 2015). However, the role of LRP-1 in cerebrovascular endothelial cell IR-β regulation and its effects on insulin signaling have been less studied. By employing RNA interference, we found that silencing LRP-1 did affect post-translational regulation of IR-β without affecting mRNA levels (Fig. 9A&B), hence lowered IR-β levels, and impaired insulin-mediated activation of insulin receptor pIR (Tyr1131) and downstream signaling protein pAKT (S473) (Fig. 9C–G). Surprisingly however, IR-β silencing showed no effect on LRP-1 mRNA or protein levels but diminished insulin-mediated IR and AKT activation (Fig. 9C–G). These findings suggest the presence of an imperative cross talk between LRP-1 and IR-β at post-translational level in pBCEC, and that Aβ impairs insulin receptor dynamics and function by accelerating
IR-β and LRP-1 degradation at the BBB. Limitations of the present study may refer to the animal model used. The development of AD pathology in a transgenic, APP over-expressing AD mouse model like 3xTg-AD will not exactly mimic human pathology owing to the heterogeneity and complexity of the disease. Similarly, it is important to remember that FHD feeding is not only a model of diabetes but also of hyperlipidemia, atherosclerosis, and metabolic syndrome. Further studies using more robust models reciprocating human pathology may provide valuable insights regarding the early changes in cerebrovascular LRP-1 and IR-β receptors and their impact on the development and progression of the disease.

In conclusion, our study demonstrates impaired central and cerebrovascular IR-β and LRP-1 levels along with impaired central insulin signaling in male and female 3xTg-AD mice with early AD pathology. Changes in cerebrovascular IR-β and LRP-1 levels corresponded with elevated LC3B levels in 3xTg-AD mice. FHD feeding further exacerbated central and peripheral LRP-1 levels independent on changes in IR-β levels at the BBB. Gender variability was observed in food intake which corresponds to body weight gain, Aβ burden, and memory impairment, all elevated in female 3xTg-AD mice compared to their male counterparts. Mechanistic studies conducted in cultured pBCEC revealed that Aβ exposure enhanced IR-β and LRP-1 trafficking to lysosomes and accelerated degradation by inducing autophagy-lysosomal pathways, in turn significantly perturbing insulin mediated responses. LRP-1 deletion post-translationally affected the BBB IR-β turnover and had a significant impact on insulin-mediated responses in pBCEC. Our studies for the first time demonstrate the impact of Aβ burden on BCEC LRP-1 and IR-β homeostasis using in vitro and in vivo models.

Owing to the therapeutic importance towards drugs targeted to brain LRP-1 (Martiskainen et al., 2013; Storch and Pietrzik, 2017), intranasal insulin therapies (Aguerinos et al., 2018; Craft et al., 2012; de la Monte, 2012), and anti-diabetics (Alagiakrishnan et al., 2013; Cao et al., 2015) to modulate toxic Aβ build-up in the brain and improve memory in mild cognitively impaired AD individuals, our studies using the 3xTg-AD mouse model and in vitro porcine BCEC model elaborated Aβ mediated mechanisms leading to impairment in cerebrovascular endothelial LRP-1 and IR-β receptors which are primarily involved in Aβ uptake, insulin signaling, and cell metabolism. Targeting these receptors at the level of the BBB to modulate Aβ clearance from brain may provide possible therapeutic intervention in AD.

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Declaration of Competing Interest

There are no potential conflicts of interest relevant to this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcn.2019.103390.

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