Insulin receptor signaling mediates APP processing and β -amyloid accumulation without altering survival in a transgenic mouse model of Alzheimer's disease

Oliver Stöhr · Katharina Schilbach · Lorna Moll · Moritz M. Hettich ·
Susanna Freude · F. Thomas Wunderlich · Marianne Ernst · Johanna Zemva ·
Jens C. Brüning · Wilhelm Krone · Michael Udelhoven · Markus Schubert

Received: 3 July 2011 / Accepted: 15 October 2011 / Published online: 6 November 2011
© American Aging Association 2011

Abstract In brains from patients with Alzheimer's disease (AD), expression of insulin receptor (IR), insulin-like growth factor-1 receptor (IGF-1R), and insulin receptor substrate proteins is downregulated. A key step in the pathogenesis of AD is the accumulation of amyloid precursor protein (APP) cleavage products, β -amyloid ($A\beta$)_{1–42} and $A\beta$ _{1–40}. Recently, we and others have shown that central IGF-1

resistance reduces $A\beta$ accumulation as well as $A\beta$ toxicity and promotes survival. To define the role of IR in this context, we crossed neuron-specific IR knockout mice ($nIR^{-/-}$) with Tg2576 mice, a well-established mouse model of an AD-like pathology. Here, we show that neuronal IR deficiency in Tg2576 ($nIR^{-/-}$ Tg2576) mice leads to markedly decreased $A\beta$ burden but does not rescue premature mortality of

Oliver Stöhr, Katharina Schilbach, and Lorna Moll contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11357-011-9333-2) contains supplementary material, which is available to authorized users.

O. Stöhr · K. Schilbach · L. Moll · M. M. Hettich ·
S. Freude · J. Zemva · J. C. Brüning · W. Krone ·
M. Udelhoven · M. Schubert
Center for Endocrinology, Diabetes and Preventive
Medicine (CEDP), Center for Molecular Medicine Cologne
(CMMC), University of Cologne,
Kerpener Str 62,
50931 Cologne, Germany

S. Freude · F. T. Wunderlich · M. Ernst · J. C. Brüning ·
W. Krone · M. Udelhoven · M. Schubert
Cologne excellence cluster in Cellular Stress
Responses in Aging-Associated Diseases (CECAD),
University of Cologne,
Cologne, Germany

F. T. Wunderlich · J. C. Brüning
Max Planck Institute for Neurological Research,
Zölpricher Straße 47,
50674 Cologne, Germany

M. M. Hettich
Molecular and Cellular Cognition Lab,
German Centre for Neurodegenerative Diseases (DZNE),
53175 Bonn, Germany

M. Ernst · J. C. Brüning
Department of Mouse Genetics and Metabolism,
Institute for Genetics University of Cologne,
Zölpricher Strasse 47,
50674 Cologne, Germany

M. Udelhoven (✉) · M. Schubert (✉)
Center for Endocrinology, Diabetes and Preventive
Medicine, University of Cologne,
CMMC building 66, 5.012, Robert-Koch-Str. 21,
50931 Cologne, Germany
e-mail: michael.udelhoven@uk-koeln.de
e-mail: markus.schubert@uni-koeln.de

Tg2576 mice. Analyzing APP C-terminal fragments (CTF) revealed decreased α -/ β -CTFs in the brains of $nIR^{-/-}$ Tg2576 mice suggesting decreased APP processing. Cell based experiments showed that inhibition of the PI3-kinase pathway suppresses endosomal APP cleavage and decreases α - as well as β -secretase activity. Deletion of only one copy of the neuronal IGF-1R partially rescues the premature mortality of Tg2576 mice without altering total amyloid load. Analysis of Tg2576 mice expressing either a dominant negative or constitutively active form of forkhead box-O (FoxO)1 did not reveal any alteration of amyloid burden, APP processing and did not rescue premature mortality in these mice. Thus, our findings identified IR signaling as a potent regulator of A β accumulation in vivo. But exclusively decreased IGF-1R expression reduces AD-associated mortality independent of β -amyloid accumulation and FoxO1-mediated transcription.

Keywords Insulin receptor · Insulin-like growth factor-1 receptor · β -Amyloid · Alzheimer's disease · Tg2576 mice

Introduction

Patients suffering from type 2 diabetes (T2DM) have a two- to threefold increased relative risk for Alzheimer's disease (AD) as the most common cause of dementia (Ott et al. 1996, 1999). This might be explained by vascular complications of T2DM leading to neurodegeneration (Lovestone 1999). Alternatively, neuronal resistance for insulin/insulin-like growth factor-1 (IGF-1) might represent a molecular link between T2DM and AD, characterizing AD as a “brain type” or “type 3” diabetes (de la Monte et al. 2006; Kroner 2009; Lovestone 1999; Pilcher 2006; Steen et al. 2005). In line with this hypothesis, insulin receptor (IR) and IGF-1 receptor (IGF-1R) signaling (IIS) is markedly disturbed in the central nervous system (CNS) of AD patients (Frolich et al. 1998, 1999; Moloney et al. 2008, 2010). Detailed post-mortem investigation of brains from AD patients revealed a substantially downregulated expression of IR, IGF-1R (Rivera et al. 2005; Steen et al. 2005), and insulin receptor substrate (IRS) proteins (Moloney et al. 2010; Rivera et al. 2005; Steen et al. 2005). Similar changes in the IIS have been reported in animals fed a

high fat diet (Ho et al. 2004) suggesting that decreased IR/IGF-1R signaling might be the common molecular basis of both, T2DM and AD. In contrast, type 2 diabetic patients with AD accumulate less β -amyloid (A β) compared with non-diabetic AD patients (Craft 2009) raising the question, whether changes in IIS are cause, consequence, or maybe even compensatory counterregulation to disease.

The probably most important pathological hallmark of AD is A β accumulation. A β consists of different peptides evolving from cleavage of the amyloid precursor protein (APP), mainly occurring in two lengths: A β _{1–40} and A β _{1–42}. These peptides are prone to aggregate and particular the A β oligomers are cytotoxic and most likely the molecular basis of neurodegeneration in AD (Crews and Masliah 2010).

In *Caenorhabditis elegans*, the DAF-2 pathway (ortholog to the mammalian IIS) controls longevity (Finch and Ruvkun 2001) in a DAF-16 dependent manner. DAF-16 encodes a forkhead transcription factor (Lin et al. 1997; Ogg et al. 1997), which translocates into the nucleus (Lee et al. 2001) and modulates transcription when DAF-2 signaling is abrogated. Cohen and coworkers showed that knocking down DAF-2 in *C. elegans* expressing A β _{1–42} reduces A β toxicity (Cohen et al. 2006). This effect was mediated by two downstream transcription factors, DAF-16, and HSF-1 facilitating two different molecular mechanisms of detoxification: The first detoxification pathway leads to disaggregation and degradation of the toxic oligomers. The second mechanism mediates the formation of low toxic, high molecular weight aggregates from highly toxic small molecular weight aggregates, positively regulated by DAF-16 (A β hyperaggregation). Recently, A β hyperaggregation has been observed as cytoprotective mechanism in IGF-1 resistant mouse models of AD (Cohen et al. 2009) suggesting that Insulin/IGF-1→forkhead box-O (FoxO) transmitted signals influence A β proteotoxicity and possibly thereby survival. Thus, IIS in the brain might represent a regulatory pathway of protein turnover and aggregation, which determines lifespan, at least in models of certain neurodegenerative diseases.

In order to test this hypothesis, we generated mice deficient for either both copies of the neuronal IR or one copy of the neuronal IGF-1R as well as mice expressing a dominant negative or a constitutively

active mutant of FoxO1. These mice were crossed with Tg2576 mice, a well-established mouse model of an AD-like pathology overexpressing the Swedish mutant of APP. In rodents and humans, increased APP expression is associated with decreased life expectancy and an AD-like pathology suggesting that pathways influencing A β production or A β toxicity might lead to increased lifespan and/or altered A β accumulation/A β aggregation.

Here, we show that neuronal IR deficiency leads to decreased A β accumulation at least partially caused by decreased APP processing, but without influencing survival of Tg2576 mice. In contrast, haploinsufficiency for the IGF-1R significantly improved survival of Tg2576 mice but did not diminish A β burden. However, neither FoxO mutant rescued the premature mortality of Tg2576 mice nor altered total A β accumulation suggesting that the effects mediated via IR or IGF-1R are independent of FoxO1.

Material and methods

Animals, breeding, and genotyping

Tg2576 mice overexpressing the Swedish mutation of human APP₆₉₅ (APP^{SW}) were purchased from Taconic Corporate (Hudson, NY, USA) in a B6/SJL background. Since the genetic background of Tg2576 mice might influence mortality (Carlson et al. 1997), we used the APP^{SW} model from Taconic in a B6/SJL background and crossed these mice back for three generations in a C57BL/6 background. Due to this approach, we obtained in all four intercrossed strains (nIR^{-/-}, nIGF-1R^{+/-}, FoxO1ADA, and FoxO1DN) similar mortality rates of Tg2576 mice as described in the literature avoiding excessive mortality (El Khoury et al. 2007; Matsubara et al. 2003; Nathan et al. 2005). IGF-1R^{lox/+} and IR^{lox/lox} mice were generated and genotyped as described previously (Bruning et al. 2000; Stachelscheid et al. 2008) and crossed with synapsin-Cre (Syn-Cre) mice to achieve neuron-specific deletion. Mice which did not express APP^{SW} or Syn-Cre served as controls. nIR^{-/-}, nIGF-1R^{+/-}, FoxO1ADA, FoxO1DN, and Syn-Cre mice used for breeding were all on a pure C57BL/6 background.

Two transgenic mice expressing different FoxO1 mutants have been generated: (1) FoxO1ADA, which is constitutively nuclear localized due to mutation of

T24 and S316 to A and S253 to D (Supplementary Fig. 1a). The cDNA of the FoxO1ADA mutant was subcloned into the *rosa26* locus. Since this locus is ubiquitously expressed, the sequence of the FoxO1ADA is separated by a loxP sites flanked stop cassette (Supplementary Fig. 1b). (2) FoxO1DN (Supplementary Fig. 1a), which is transactivation domain deleted and acts dominant negative. This mutant was cloned into the *rosa26* locus as well (Supplementary Fig. 1b). In order to get specific neuronal expression, both FoxO1 mutant strains were crossed with Syn-Cre mice.

Animals were housed in a 12-h light–dark cycle (07:00 on and 19:00 off) and were fed a standard rodent diet (89% dry matter, 22.5% crude protein, 5% crude fat, 4.5% crude fibre, 6.5% crude ash, 50.5% nitrogen free extracts, and a standard amount of different minerals, amino acids, vitamins, and trace elements; breeding diet #1314, Altromin, Lage, Germany). All animal procedures were performed in accordance with the German Laws for Animal Protection and were approved by the local animal care committee and the Bezirksregierung Köln.

Immunoblotting

Brains were lysed in buffer (50 mM Hepes (pH 7.4), 50 mM NaCl, 1% Triton X-100, 10 mM EDTA, 0.1 M NaF, 17 μ g/ml Aprotinin, 2 mM Benzanidine, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, and 10 mM Na₃VO₄) using a polytron. Protein expression was determined from whole-brain lysates (50–100 μ g) dissolved in Laemmli buffer and resolved on 7.5%, 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were transferred to PVDF; membranes were blocked with 5% Western blot blocking solution and incubated with the appropriate antisera.

Following primary antibodies were used: anti-insulin receptor β -subunit, anti-A disintegrin and metalloprotease-10/-17 (ADAM-10/-17), anti-flotillin-1 (C-2), anti-Rab5 (D-11), anti-TACE (C15; Santa Cruz Biotechnology, CA, USA), anti-actin (MP Biomedicals, Ohio, USA), anti- β -site APP cleaving enzyme (BACE), A β , anti-insulin-degrading enzyme (IDE; Chemicon International/Upstate/Millipore, MA, USA), anti-protein kinase B (PKB/AKT), anti-phospho-

PKB^{Ser473} (AKT), anti-extracellular regulated kinase (ERK)-1/-2, anti-phospho-ERK-1/-2^{Thr202/Tyr204}, anti-glycogen synthase kinase (GSK)-3 α / β , anti-GSK-3 β , anti-phospho-GSK-3 α / β ^{Ser9/Ser21}, anti-APP, anti-IGF-1R (Cell Signaling Technology Inc, MA, USA), anti- α / β C-terminal fragments (α / β CTF; Sigma-Aldrich, Munich, Germany), anti-6E10 ($A\beta_{1-17}$; Covance, NJ, USA), anti-presenilin 1 (Invitrogen Corporation, Carlsbad, CA, USA), anti-eGFP (D51; Cell signaling Technology Inc, MA, USA), anti-FoxO1 (L27, 29H4; Cell Signaling Technology Inc, MA, USA). Detection of $A\beta$ oligomers was performed as described previously (Lesne et al. 2006). For dot blot analysis, samples used for the Western blot analysis (10 μ g total protein) were directly applied to the membrane, air dried, and blocked with 5% nonfat milk followed by incubation with antibody A11 (Invitrogen), an antibody that specifically recognizes the oligomeric form of $A\beta$. Secondary antibodies anti-Mouse-IgG and anti-Rabbit-IgG were purchased from Sigma-Aldrich, Munich, Germany.

Metabolic characterization, glucose, and insulin tolerance tests

Mice were weighed weekly beginning at weaning in week 3 until performance of glucose and insulin tolerance tests in weeks 10 and 11. From week 12, blood glucose and weight were measured every 4 weeks.

For insulin tolerance tests animals were starved overnight (16 h) and injected with 0.75 U/kg body weight of human insulin (Novo Nordisk, Copenhagen, Denmark) into the peritoneal cavity. Blood glucose levels were measured in blood collected from the tail tip immediately before, and 15, 30, and 60 min after injection. Blood glucose measurements were performed using a blood glucose meter (GlucoMen, A. Menarini diagnostics, Berlin-Chemie, Neuss, Germany). Results were expressed as percentage of initial blood glucose concentration.

For glucose tolerance tests mice were starved overnight (16 h). Animals were injected with glucose (2 g/kg body weight) into the peritoneal cavity. Glucose levels were determined in blood collected from the tail tip immediately before, and 15, 30, 60, and 120 min after the injection using a glucose meter.

Secretase assays

α - and β -secretase assays were performed with SensoLyte 520 TACE Activity Fluorimetric Assay Kit and SensoLyte beta-Secretase Fluorimetric Assay Kit as described by the manufacturer (Anaspec). Twenty micrograms of protein from crude cell lysate homogenate were used per experiment. Experiments were done in duplicates with $n=3$. Measurements were performed in 96-well plates (nunc) in a Mithras LB 940 platereader (Berthold Technologies). β -Secretase activity of freshly isolated hippocampi was measured following the manufacturers protocol at 535-nm emission (#FP002, R&D Systems Inc, Minneapolis, MN, USA).

β -Amyloid₁₋₄₀ and β -amyloid₁₋₄₂ ELISA

Amyloid was extracted using 5 M guanidine HCl in 50 mM Tris-HCl, pH 8.0. Then ELISAs of $\beta A_{1-40/1-42}$ were performed following the manufacturers protocol (#KHB3481/3441, Invitrogen Corporation, Carlsbad, CA, USA).

Sucrose gradient

We used 5 mg cell lysate to perform one sucrose gradient. Cells were grown on 15 cm cell culture plates and were lysed in 1% Triton X-100 (Appl-chem) in Tris-buffered saline (TBS) containing complete protease inhibitor (Roche). Cells were homogenized in a loose fitting potter with 40 strokes. Cell debris and nuclei were pelleted afterwards (1,000 g, 10 min, 4°C). Protein concentration was measured using Bradford assay and the volume containing 5 mg of protein was adjusted to 500 μ l with lysis buffer. Final lysate was mixed with an equal volume of 85% sucrose in TBS. The mix was overlaid with 3 ml 35% and 1 ml 5% sucrose in 5 ml centrifuge tubes (Beckman). After centrifugation at max. 240,000 $\times$ g for 17 h (Ti55 rotor, Beckman Ultracentrifuge), we took 500- μ l fractions from top to bottom. All steps were performed at 4°C.

For secretase inhibition the following inhibitors were used: α -secretase 20 μ M TAPI-2 (Enzo Life-Sciences, Loerrach, Germany), β -secretase 10 μ M β -secretase inhibitor II (BioVision Research Products, California USA), γ -secretase 0.2 μ M III-31C (Sigma-

Aldrich, Munich, Germany). For inhibition of PI3K LY 294002, 100 μ M was used.

Statistical analysis

To quantify the changes in optical density, we used the software AIDA (Version 4.00.027, Raytest, Straubenhardt, Germany). For statistical analysis of the different study groups unpaired Student's *t* test was performed. Statistical significance was defined as $*p < 0.05$. For Kaplan–Meier analysis, the XLSTAT-Life software, a Microsoft Excel add-in (www.xlstat.com) was used. For comparison of the different study groups, Wilcoxon rank tests were performed. Statistical significance was defined as $*p < 0.05$.

Results

Neuronal insulin receptor deletion does not influence survival of Tg2576 mice

Recent data suggest that brain-specific IGF-1 resistance increases longevity (Freude et al. 2009; Kappeler et al. 2008; Taguchi et al. 2007). Furthermore, decreased IGF-1 signaling reduces A β toxicity in worms and mice (Cohen et al. 2006, 2009). Currently, there are limited data available elaborating the role of neuronal IR signaling in respect to longevity and A β pathology in vivo.

To further elucidate the role of the IR in this context, we crossed neuronal specific IR-deficient mice (nIR^{-/-}) with mice expressing the Swedish mutation of human APP₆₉₅ containing the double mutation Lys⁶⁷⁰→Asn, Met⁶⁷¹→Leu which was found in a Swedish family with early-onset AD (APP^{SW}, Tg2576 mice) (Hsiao et al. 1996). Tg2576 mice develop an age-dependent memory impairment, A β accumulation and form extracellular A β plaques beginning at the age of 6–8 months (Stein and Johnson 2002). Furthermore, these mice display an increased mortality (Freude et al. 2009). To achieve neuron-specific deletion, we crossed mice carrying a floxed exon 4 of the IR gene with mice expressing the Cre recombinase under control of the Syn-Cre. Previous investigations of our group have shown that using these mice, Cre-recombinase-mediated deletion of floxed DNA sequences occur predominantly in dentate gyrus, CA1-3 region of the hippocampus,

amygdala, and with lower expression in piriform cortex, neocortex and thalamus (Freude et al. 2009). Our current analysis using transgenic mice expressing eGFP after Cre-mediated deletion of a floxed stop sequence confirmed this expression pattern (Supplementary Fig. 1c). Accordingly, Western blot analysis of isolated hippocampi revealed a reduction of IR expression and in line with neuronal insulin resistance decreased response to insulin stimulation but unaltered basal Akt and Erk-1/-2 phosphorylation (Fig. 2a, f; Supplementary Fig. 2). However, in contrast to IRS-2^{-/-}Tg2576 and nIGF-1R^{-/-}Tg2576 mice (Freude et al. 2009) in which survival was prolonged due to whole body knockout of IRS-2 or neuronal IGF-1R, neuron-specific IR deletion in a Tg2576 background did not rescue premature lethality of Tg2576 mice (El Khoury et al. 2007; Leissring et al. 2003) in both genders (Fig. 1a). To exclude any influence of impaired glucose and/or insulin sensitivity, glucose and insulin tolerance tests were performed in all genotypes revealing unaltered glucose metabolism in these mice (Fig. 1b). However, since ITTs and GTTs represent only one time point during the 60 weeks study period, we monitored our experimental animals concerning body weight and blood glucose levels monthly revealing no differences in blood glucose levels and body weight between nIR^{-/-}Tg2576 and Tg2576 mice (Supplementary Fig. 3).

Neuronal insulin receptor deletion reduces A β accumulation in Tg2576 mice

Since increased A β accumulation in the CNS might be a contributing factor of premature lethality in Tg2576 mice (El Khoury et al. 2007; Leissring et al. 2003), we next investigated A β accumulation. A β peptides are proteolytically released from the APP via sequential cleavage by two aspartyl proteases, the β - and γ -secretase. The cleavage products mainly occur in two lengths, A β _{1–40} and A β _{1–42}. Previous work of our group has shown that nIGF-1R^{-/-}Tg2576 mice accumulate less A β compared with Tg2576 mice (Freude et al. 2009). Reduced occurrence of α/β CTF in hippocampal lysates from these mice suggests decreased APP processing probably due to decreased β -secretase activity. We therefore investigated whether the amount of APP cleavage products was also affected in nIR^{-/-}Tg2576 mice. Western blot analysis revealed comparable transgenic APP levels in Tg2576 and

Fig. 1 Kaplan–Meier analysis and metabolic characterization of nIR^{-/-}Tg2576 mice and respective controls up to 60 weeks of age. **a** Kaplan–Meier analysis. Survival was assessed from 172 female (86 wild type, 50 Tg2576, 20 nIR^{-/-}, and 16 nIR^{-/-}Tg2576) and 193 male mice (105 wild type, 52 Tg2576, 22 nIR^{-/-}, and 14 nIR^{-/-}Tg2576). **p*<0.05 (Wilcoxon rank test). **b** Glucose tolerance tests (GTT) of mice aged 10–

11 weeks. GTT was assessed from 88 female (45 wild type, 15 Tg2576, 19 nIR^{-/-}, and 9 nIR^{-/-}Tg2576) and 100 male mice (50 wild type, 22 Tg2576, 19 nIR^{-/-}, and 9 nIR^{-/-}Tg2576). Insulin tolerance tests (ITT) of mice aged 10–11 weeks. ITT was assessed from 85 female (43 wild type, 14 Tg2576, 19 nIR^{-/-}, and 9 nIR^{-/-}Tg2576) and 103 male mice (51 wild type, 25 Tg2576, 18 nIR^{-/-}, and 9 nIR^{-/-}Tg2576)

nIR^{-/-}Tg2576 mice (Fig. 2a, b). Interestingly, brains of 60 weeks old nIR^{-/-}Tg2576 mice showed significantly less A β accumulation in Western blot analysis from hippocampus lysates compared with Tg2576 mice (Fig. 2a). These findings were further confirmed by ELISA analysis detecting A β _{1–40} and A β _{1–42} (Fig. 2c) revealing a significant decrease of A β _{1–40/42} peptides in hippocampi from nIR^{-/-}Tg2576 compared with Tg2576 mice at 60 weeks. Furthermore, A β oligomers occurred at lower concentrations in nIR^{-/-}Tg2576 compared with Tg2576 brains (Fig. 2d, e). Since the reduced A β accumulation was present despite unchanged mortality of nIR^{-/-}Tg2576 compared with Tg2576 mice, these data indicate that neuron-specific insulin receptor deletion prevents A β accumulation but does not prolong survival.

Previous investigations demonstrated that IR/IGF-1R-mediated signals increase APP α - and β -cleavage in cultured cells (Adlerz et al. 2007; Costantini et al. 2006). We therefore determined the abundance of β CTF (C-terminal fragment) and α CTF in the CNS of nIR^{-/-} mice and respective controls (Fig. 2a). Interestingly, α / β CTFs were reduced in nIR^{-/-}Tg2576 compared with Tg2576 mice, while APP levels were unchanged (Fig. 2a). These data indicate that the reduced A β accumulation observed in nIR^{-/-}Tg2576

mice might at least partially result from decreased APP processing (Fig. 2a).

In order to analyze whether reduced expression of the different secretases accounts for altered APP cleavage in nIR^{-/-}Tg2576 mice, we investigated protein levels of the BACE-1 which acts as a β -secretase catalyzing the rate-limiting step of A β generation (Fig. 2f). However, since no changes of BACE-1 expression were detectable comparing nIR^{-/-}Tg2576 mice and Tg2576 mice, BACE-1 activity might be reduced in nIR^{-/-}Tg2576 mice compared with Tg2576 mice.

PI3-Kinase signaling regulates endosomal APP processing

To further analyze the role of the IIS in endogenous APP processing, we isolated endosomes and lipid rafts from wild-type human neuroblastoma cells (SHSY5Y) as well as SHSY5Y cells stably expressing siRNA against IRS-2 using sucrose density gradient centrifugation. siIRS-2 cells express about 75% less IRS-2 compared with wild-type SHSY5Y (Fig. 3a). As quality control of membrane fraction preparations, we used Rab-5 antibodies to identify endosomal fractions, and flotillin antibodies to detect lipid rafts. As shown in Fig. 3b, flotillin-positive lipid

Fig. 2 Amyloid metabolism of nIR^{-/-}Tg2576 and respective control mice aged 60 weeks. **a** Western blot analysis of insulin receptor β (*IRβ*), amyloid precursor protein (*APP*), Aβ, αCTF, βCTF, and RasGAP. **b** Quantitative analysis of APP expression in hippocampi. *n*=6 per group. **c** ELISA of β-amyloid_{1-40/1-42} peptides from Aβ-guanidine-HCl extractions of hippocampi. *n*=4–6 per group. Data represent means±SEM, **p*<0.05 (Student's *t* test). **d** Western blot analysis of Aβ oligomers

typical example of three independent experiments. **e** Dot blot analysis of the TBS-soluble fraction of hippocampal lysates (10-μg total protein per dot) from Tg2576 and nIR^{-/-}Tg2576 using the oligomer specific antibody A11. The figure shows typical results from the same membran. *n*=3 independent experiments. **f** Western blot analysis of BACE-1, Akt, pAkt^{ser473}, Erk-1/-2 and pErk-1/-2^{Thr202/Tyr204}, and actin

rafts are found mainly in fractions 2 and 3, whereas Rab-5-positive endosomes occur predominantly in fractions 8 and 9.

As described previously, we detected APP CTFs exclusively in the endosomal fraction of wild-type SHSY5Y (Fig. 3b) (Marks and Berg 2010). Surprisingly, CTF appearance is completely abolished after treatment with the PI3K inhibitor LY294002 (Fig. 3b). To further elucidate the role of IRS-2 in formation of CTFs, we analyzed siIRS-2 cells with and without LY294002 treatment. Interestingly, CTF expression in untreated siIRS-2 cells is reduced compared with untreated wild-type cells (Fig. 3b). However, treatment with the PI3-kinase inhibitor LY294002 completely blocks CTF generation (Fig. 3b) whereas APP expression and trafficking remained unchanged (Fig. 3c). These findings suggest that PI3K activated by IRS-2 promotes CTF formation. Previous work indicated that endosomal APP

procession is mainly mediated via α-secretase activity (Marks and Berg 2010). Therefore, we analyzed the effect of α-, β-, and γ- secretase inhibitors on endogenous CTF formation and protein abundance of BACE-1, ADAM-10, and TACE in the different membrane fractions (Fig. 3d). Interestingly, only the α-secretase inhibitor TAPI-2 (Enzo LifeSciences, Lörrach, Germany, 20 μM final concentration) reduced CTF formation whereas the β-secretase inhibitor II, BioVision Research Products, California, USA) and γ-secretase inhibitor (III-31C, Sigma, 0.2 μM final concentration) had no effect (Fig. 3c) suggesting that endosomal APP procession is mediated mainly via the two possible α-secretases ADAM-10 and/or TACE (Jacobsen et al. 2010). However, Western blot analysis of BACE-1, ADAM-10, and TACE did not show any differences between wild-type and siIRS-2 cells (Fig. 3e). Since the PI3K inhibitor LY294002

Fig. 3 PI3K inhibition and APP C-terminal fragment formation in endosomes. **a** Western blot analysis of SHSY5Y wild-type and siIRS-2 cells using anti-IRS-2 antibodies. **b** Western blot analysis of APP CTFs from membrane fractions separated via sucrose gradient density centrifugation of wild-type, siIRS-2, and LY294002-treated cells. To test quality of prepared fractions, Western blots for marker proteins identifying lipid rafts (flotillin) and endosomes (Rab5) were performed. **c** Western blot analysis of APP from membrane fractions separated via sucrose gradient density centrifugation of SHSY5Y and siIRS-2 cells treated with or without LY294002.

inhibits CTF formation in wild-type SHSY5Y and siIRS-2 cells, we treated SHSY5Y cells with LY294002 or control medium followed by α -/ β -secretase activity measurements. At all time points α -

as well as β -secretase activity was significantly reduced in PI3K inhibitor treated SHSY5Y cells compared with controls (Fig. 3f). Thus, PI3K mediates activation of α -/ β -secretase explaining at least

partially the reduced occurrence of APP CTFs in insulin and IGF-1 resistant brains of Tg2576 mice. In order to confirm that IR/IGF-1R signaling is involved in regulating β -secretase activity in vivo, we isolated hippocampi from mice being neuron specifically deficient for the IR (nIR) or the IGF-1R (nIGF-1R) and measured β -secretase activity. Consistent with our in vitro data, β -secretase activity was significantly reduced in hippocampi of both conditional knockout models (Fig. 3g) suggesting that IR- and IGF-1R-mediated signals modulate β -secretase activity.

Neuron-specific deletion of one IGF-1R copy partially rescues premature mortality in Tg2576 mice

A recent study has shown that homozygous neuron-specific deletion of the IGF-1R completely rescues premature mortality of Tg2576 mice (Freude et al. 2009). Neuron-specific deletion of one IGF-1R gene copy (nIGF-1R^{+/-}) might partially rescue the premature mortality of Tg2576 mice (Freude et al. 2009). Probably due to the small sample size the difference between IGF-1R^{+/-}Tg2576 and Tg2576 mice failed to reach significance. In order to definitely answer the question whether neuron-specific heterozygosity for the IGF-1R influences survival in Tg2576 mice, we further increased the animal numbers of our initially

published nIGF-1R^{+/-} cohort. Kaplan–Meier analysis revealed that nIGF-1R^{+/-} mice are partially protected against APP^{SW}-induced lethality (Fig. 4a). Insulin and glucose tolerance tests showed unchanged glucose metabolism in all genotypes (Fig. 4b). As observed in mice with a homozygous deficiency for the IGF-1R (Freude et al. 2009) downstream IR/IGF-1R signaling proteins abundance e.g. Akt, GSK-3 β and ERK-1/-2 was unchanged and no difference in basal phosphorylation of these proteins has been detected (pAK-T^{Ser473}, pGSK-3 β ^{Ser9}, and pERK-1/-2^{Thr202/Tyr204}) in nIGF-1R^{+/-}Tg2576 compared with Tg2576 mice (Fig. 5a). However, consistent with IGF-1 resistance isolated hippocampi of nIGF-1R^{+/-} mice express slightly less IGF-1R and respond less to IGF-1 stimulation compared with WT (Supplementary Fig. 4). Furthermore, expression of apolipoprotein E (ApoE), and IDE was unaltered in hippocampal lysates of nIGF-1R^{+/-}Tg2576 compared with Tg2576 mice (Fig. 5a).

Heterozygosity of IGF-1R in neurons does not alter total A β burden but decreases A β oligomers in APP^{SW} overexpressing mice

Analysis of α - and β CTF in hippocampi from nIGF-1R^{+/-}Tg2576 mice aged 60 weeks (Fig. 5b) revealed

Fig. 4 Kaplan–Meier analysis and metabolic characterization of nIGF-1R^{+/-}Tg2576 mice and respective controls up to 60 weeks of age. **a** Kaplan–Meier analysis. Survival was assessed from 229 female (117 wild type, 72 Tg2576, 28 nIGF-1R^{+/-}, and 12 nIGF-1R^{+/-}Tg2576) and 263 male mice (152 wild type, 60 Tg2576, 33 nIGF-1R^{+/-}, and 18 nIGF-1R^{+/-}Tg2576). * $p < 0.05$ (Wilcoxon rank test). **b** Glucose tolerance tests (GTT) of mice aged 12 weeks. GTT was assessed from 132 female (67

wild type, 36 Tg2576, 18 nIGF-1R^{+/-}, and 11 nIR^{+/-}Tg2576) and 130 male mice (72 wild type, 27 Tg2576, 18 nIGF-1R^{+/-}, and 13 nIR^{+/-}Tg2576). Insulin tolerance tests (ITT) of mice aged 12 weeks. ITT was assessed from 124 female (62 wild type, 37 Tg2576, 16 nIGF-1R^{+/-}, and 9 nIR^{+/-}Tg2576) and 119 male mice (71 wild type, 23 Tg2576, 15 nIGF-1R^{+/-}, and 10 nIR^{+/-}Tg2576)

Fig. 5 IR/IGF-1R signaling and amyloid metabolism of nIGF-1R^{+/-}Tg2576 and respective control mice aged 60 weeks. **a** Western blot analysis of ApoE, IRS-1, IDE, pAkt^{Ser473}, pGSK-3β^{Ser9}, pErk-1/-2^{Thr202/Tyr204}, and respective loading controls. **b** Western blots of hippocampal lysates for amyloid precursor protein (APP), IR, C-terminal fragment-α (αCTF), and βCTF, *n*=4 per group. **c** Western blot analysis of Aβ oligomers using

β-amyloid₁₋₁₇ antibodies. **d** ELISA of β-amyloid_{1-40/1-42} peptides from Aβ-guanidine-HCl extractions of hippocampi. *n*=4 per group. Data represent means±SEM. Age matched animals without APP^{sw} and/or Syn-Cre expression served as controls. **p*<0.05 (Student's *t* test). **e** Quantification of APP expression in hippocampi of nIGF-1R^{+/-}Tg2576 and Tg2576 mice. *n*=6 per group

a slight but visible reduction of α and βCTF in the hippocampus while total APP expression remained unchanged (Fig. 5b, e). In contrast to nIR^{-/-}Tg2576 (Fig. 2b) and nIGF-1R^{+/-}Tg2576 (Freude et al. 2009) mice, hippocampi of nIGF-1R^{+/-}Tg2576 mice showed no reduction of total Aβ burden even in 60 weeks old mice (Fig. 5c, d). According to previous studies in IGF-1-resistant models (Cohen et al. 2006, 2009), low molecular weight Aβ oligomers occur less in nIGF-1R^{+/-}Tg2576 mice compared with Tg2576 mice (Fig. 5c) possibly suggesting Aβ hyperaggregation as molecular mechanism of detoxification in these mice (Cohen et al. 2006, 2010, 2009). However, the reduced Aβ load and low molecular weight Aβ oligomer concentration in the absence of a survival benefit in nIR^{-/-}Tg2576 mice might argue against a predominant role of Aβ toxicity as leading cause of death in Tg2576 mice. Our data point towards a role of IGF-1 resistance in mediating cellular stress defense which is not transmitted via decreased IR signaling.

FoxO expression in the rodent brain

FoxO transcription factors act as downstream target of the IIS pathway. So far, there are four different members of the mammalian FoxO-family known: FoxO1, FoxO3a, FoxO4, and FoxO6. Apart from FoxO4, all of them are expressed in the murine brain (Hoekman et al. 2006). Previous studies in worms have shown that lifespan is determined by the IIS pathway and this effect is predominantly mediated via the neuronal IIS (Kenyon et al. 1993; Wolkow et al. 2000). Data obtained in *C. elegans* suggest that the Aβ hyperaggregation observed in IGF-1 resistant models is induced via FoxO-mediated transcription.

So far, it is unclear which neuronal subtype or formation of the mammalian brain plays the key role in controlling lifespan, although the hippocampus is proposed to take a leading role (Freude et al. 2009). Up to now little is known about expression and function of the single FoxOs expressed in mammalian

brain. Since antibodies for the different FoxO transcription factors are of limited quality, we decided to probe different brain regions (i.e., cerebellum, thalamus, hippocampus, frontal cortex, parietal cortex, and occipital cortex) for FoxO1, FoxO3a, and FoxO6 using real-time PCR and TaqMan Gene Expression Assays with TBP (Tata box binding protein) as housekeeping gene. In a first step, we compared the expression of the individual FoxOs in the above-mentioned brain regions and the expression of the different FoxOs in whole-brain preparations (Table 1). FoxO1 had its highest expression in the hippocampus, which was significant compared with whole brain and the other brain regions apart from the thalamus. FoxO3a was found to be significantly higher expressed in frontal and parietal cortex compared with the overall expression in whole-brain lysates. FoxO6 mRNA levels were significantly lower in thalamus compared with whole brain.

In a second step, we normalized expression of FoxO3a and FoxO6 to FoxO1 (Table 2).

Overall FoxO1 and FoxO3 expression in whole brain was significantly higher than the expression of FoxO6. In the hippocampus, FoxO1 mRNA concentration was 1.6-fold higher compared with FoxO3a and 3.1-fold higher compared with FoxO6 mRNA levels. In the different cortical regions, FoxO3a was higher expressed than the other FoxOs reaching significance for parietal and occipital cortex compared with both, FoxO1 and FoxO6. Thus, FoxO1 seems to be the major FoxO transcription factor in the hippocampus, whereas FoxO3 seems to be the predominant FoxO species in the cortex. Since our genetic approach mainly alters expression of IR or IGF-1R in the hippocampus and FoxO1 seemed to be the predominant FoxO variant in this particular region, we decided to investigate the role of FoxO1

in the context of APP processing, A β accumulation, and APP^{sw}-induced mortality.

We therefore created two different mouse lines expressing neuron-specific two different mutants of the FoxO1 transcription factor. The first mutant is FoxO1ADA, which is constitutively nuclear expressed due to mutation of T24 and S316 to A and S256 to D. The cDNA of the FoxO1ADA mutant was subcloned into the *rosa26locus*. Since this locus is ubiquitously expressed, the sequence of the FoxO1ADA was separated by a loxP sites flanked stop cassette (so called FoxO1ADA mice). Furthermore, the FoxO1ADA cDNA is followed by an eGFP sequence separated via an internal ribosome entry site (for targeting strategy, see Supplementary Fig. 1). The second FoxO1 mutant is transactivation domain deleted (FoxO1DN) and acts dominant negative. This mutant was cloned into the *rosa26locus* using the same targeting strategy (Supplementary Fig. 1). In order to get neuron-specific expression, these mice were crossed with Syn-Cre mice.

Neuron-specific FoxO1ADA as well as FoxO1DN expressing animals were viable and lived at least 90 weeks (data not shown). We crossed both the FoxO1ADA and FoxO1DN mice into a Tg2576 background and analyzed glucose metabolism, A β -pathology, and mortality.

Expression of a constitutively active FoxO1 mutant does not rescue mortality or A β burden of Tg2576 mice

To verify FoxO1ADA protein expression in hippocampi of the respective genotypes, Western blot analysis with antibodies against FoxO1ADA and eGFP were performed. Expression of eGFP was, as expected, only

Table 1 Determination of FoxO1, FoxO3a, and FoxO6 expression in murine brain using real-time PCR

	16 weeks	FoxO1		FoxO3a		FoxO6	
		x-fold $\pm$ SEM	<i>p</i>	x-fold $\pm$ SEM	<i>p</i>	x-fold $\pm$ SEM	<i>P</i>
Whole brain		1.0 $\pm$ 0.04		1.0 $\pm$ 0.03		1.0 $\pm$ 0.22	
Hippocampus		1.8 $\pm$ 0.27	0.018	1.1 $\pm$ 0.18	0.490	1.0 $\pm$ 0.49	0.931
Thalamus		1.0 $\pm$ 0.25	0.898	1.0 $\pm$ 0.05	0.850	0.5 $\pm$ 0.09	0.037
Frontal cortex		1.1 $\pm$ 0.15	0.643	1.3 $\pm$ 0.13	0.050	0.9 $\pm$ 0.34	0.794
Parietal cortex		0.9 $\pm$ 0.08	0.345	1.6 $\pm$ 0.27	0.047	0.6 $\pm$ 0.22	0.172
Occipital cortex		0.9 $\pm$ 0.09	0.255	1.4 $\pm$ 0.19	0.073	0.9 $\pm$ 0.27	0.690

Expression of FoxO1, FoxO3a, and FoxO6 RNA normalized to the respective whole-brain lysate. *n*=6 per brain region and FoxO (age, 16 weeks)

Table 2 Determination of FoxO1, FoxO3a, and FoxO6 expression in murine brain using real-time PCR

16 weeks	FoxO1	FoxO3a		FoxO6		
	<i>x</i> -fold $\pm$ SEM	<i>x</i> -fold $\pm$ SEM	<i>p</i> vs. FoxO1	<i>x</i> -fold $\pm$ SEM	<i>p</i> vs. FoxO1	<i>p</i> vs. FoxO3a
Whole brain	1.0 $\pm$ 0.04	1.0 $\pm$ 0.03	0.602	0.5 $\pm$ 0.11	0.002	0.003
Hippocampus	1.0 $\pm$ 0.16	0.6 $\pm$ 0.11	0.068	0.3 $\pm$ 0.14	0.009	0.096
Thalamus	1.0 $\pm$ 0.27	1.0 $\pm$ 0.05	0.867	0.2 $\pm$ 0.04	0.019	0.001
Frontal cortex	1.0 $\pm$ 0.15	1.2 $\pm$ 0.12	0.356	0.5 $\pm$ 0.17	0.026	0.004
Parietal cortex	1.0 $\pm$ 0.09	1.7 $\pm$ 0.29	0.037	0.3 $\pm$ 0.12	0.001	0.001
Occipital cortex	1.0 $\pm$ 0.10	1.5 $\pm$ 0.21	0.047	0.5 $\pm$ 0.15	0.027	0.003

Expression of FoxO3a and FoxO6 RNA normalized to FoxO1 expression in the respective brain region. *n*=6 per brain region and FoxO (age, 16 weeks). **p*<0.05 (Student's *t* test)

detectable in the genotypes FoxO1ADA and FoxO1A-DATg2576 (Fig. 6c). Surprisingly, FoxO1A-DATg2576 mice which were supposed to mimic the IGF-1 resistant state on FoxO1-mediated transcription showed a tendency to die even earlier than Tg2576 mice (females, *p*=0.1; males, *p*<0.001; all, *p*=0.072) (Fig. 6a). During a breeding period of 3 years, we were able to generate only two male FoxO1A-DATg2576 mice which died within the first 2 months after birth. However, this phenotype is much less pronounced in females (Fig. 6a).

To exclude any influence of possibly affected glucose metabolism, insulin and glucose tolerance tests were performed which revealed no differences between FoxO1ADATg2576 and Tg2576 mice (Fig. 6b). During the study period of 60 weeks, body weight and blood glucose levels in female mice remained unchanged (Supplementary Fig. 6) whereas the only two male FoxO1ADATg2576 mice were significantly smaller and had lower blood glucose levels compared with Tg2576 littermates (Supplementary Fig. 6).

Basal expression and phosphorylation of key mediators of the IR/IGF-1R signaling cascade were unchanged in all genotypes (Supplementary Fig. 6c).

Protein expression of APP as well as the cleavage products β CTF and α CTF did not differ between FoxO1ADATg2576 and Tg2576 mice (Fig. 6d). Also, total A β load and occurrence of A β amyloid oligomers analyzed in the SDS-soluble fraction remained unchanged in the respective genotypes (Fig. 6e, f). These data suggest that FoxO1-mediated transcription in FoxO1ADATg2576 mice does not explain the phenotypes observed either in nIGF-1R^{+/−}Tg2576, nIGF-1R^{−/−}Tg2576 (Freude et al. 2009), or nIR^{−/−}Tg2576 mice.

Expression of a dominant negative FoxO1 mutant does not influence mortality or A β burden of Tg2576 mice

To determine whether inhibition of FoxO1-mediated transcription has any effects in this context, we generated Tg2576 mice expressing the FoxO1DN mutant. FoxO1DN mice display mutant FoxO1 (FoxO1DN) protein expression in hippocampi of the respective genotypes as shown by Western blot analysis using antibodies against FoxO1 and eGFP. Expression of FoxO1DN and eGFP as expected was only detectable in FoxO1DN and FoxO1DNTg2576 (Fig. 7c) mice.

Kaplan–Meier analysis of dominant negative FoxO1 mutants in a Tg2576 background revealed no difference in mortality of Tg2576 and FoxO1DNTg2576 mice (Fig. 7a). Thus, no protection against the premature mortality of Tg2576 mice was detected.

To exclude any influence of altered glucose metabolism, insulin and glucose tolerance tests were performed which revealed no differences in glucose metabolism between FoxO1DNTg2576 and Tg2576 mice (Fig. 7b). Furthermore, body weight and blood glucose levels in both genders (Supplementary Fig. 7a, b) remained unchanged during the whole study. Protein expression and phosphorylation of Akt and Erk-1/-2 showed no difference between the genotypes (Supplementary Fig. 7c).

Protein expression of APP as well as the cleavage products β CTF and α CTF did not differ between FoxO1DNTg2576 and Tg2576 mice (Fig. 7d). Total A β load and A β amyloid oligomers concentrations in the SDS-soluble fraction remained unchanged in the Tg2576 and FoxO1DNTg2576 hippocampi (Fig. 7e, f).

Fig. 6 Kaplan–Meier analysis and metabolic characterization of FoxO1ADATg2576 mice and respective controls up to 60 weeks of age. **a** Kaplan–Meier analysis. Survival was assessed from 235 female (122 wild type, 77 Tg2576, 22 FoxO1ADA, and 14 FoxO1ADATg2576) and 229 male mice (128 wild type, 66 Tg2576, 33 FoxO1ADA, and 2 FoxO1ADATg2576). $*p < 0.05$ (Wilcoxon rank test). **b** Glucose tolerance tests (GTT) of mice aged 10–11 weeks. GTT was assessed from 78 female (20 wild type, 24 Tg2576, 21 FoxO1ADA, and 13 FoxO1ADATg2576) and 68 male mice (29 wild type, 13 Tg2576, 23 FoxO1ADA, and 2 FoxO1ADATg2576). Insulin tolerance tests (ITT) of mice aged 10–11 weeks. ITT was assessed from 82 female (21 wild type, 27 Tg2576, 21

FoxO1ADA, and 13 FoxO1ADATg2576) and 75 male mice (30 wild type, 19 Tg2576, 23 FoxO1ADA, and 3 FoxO1ADATg2576). **c** Western blot analysis of FoxO1 and eGFP expression in hippocampus and cortex of FoxO1ADA mice and respective controls. **d** Western blot of hippocampal expression of amyloid precursor protein (APP), Western blot analysis of C-terminal fragment- α (α CTF), and β CTF at 60 weeks of age. **e** Western blot analysis of A β oligomers using β -amyloid₁₋₁₇ antibodies at 60 weeks of age. **f** ELISA of β -amyloid_{1-40/1-42} peptides from A β -guanidine-HCl extractions of hippocampi at 60 weeks of age. $n = 4$ per group. Data represent means $\pm$ SEM. Age matched animals without APP^{sw} and/or Syn-Cre expression served as controls. $*p < 0.05$ (Student's t test)

Discussion

Increased expression of human APP in rodents (Chishti et al. 2001; Freude et al. 2009; Hsiao et al. 1995; Moechars et al. 1999; Willuweit et al. 2009), flies (Greeve et al. 2004; Rajendran et al. 2008), or humans is associated with premature mortality and an Alzheimer-like pathology (Isacson et al. 2002; Rovelet-Lecrux et al. 2006; Sleegers et al. 2006;

Wisniewski et al. 1985). There have been numerous reports in mice showing that overexpression of human APP or mutant human APP leads to increased mortality in young mice even before the onset of visible Alzheimer-like pathology or neurodegeneration (El Khoury et al. 2007; Hsiao et al. 1995). However, preventing A β production or increasing A β clearance from the brain reduces mortality in APP overexpressing mice or flies suggesting that A β

Fig. 7 Kaplan–Meier analysis and metabolic characterization of FoxO1DNTg2576 mice and respective controls up to the age of 60 weeks. **a** Kaplan–Meier analysis. Survival was assessed from 168 female (71 wild type, 48 Tg2576, 35 FoxO1DN, and 14 FoxO1DNTg2576) and 143 male mice (67 wild type, 35 Tg2576, 29 FoxO1DN, and 12 FoxO1DNTg2576). $*p < 0.05$ (Wilcoxon rank test). **b** Glucose tolerance tests (GTT) of mice aged 10–11 weeks. GTT was assessed from 118 female (38 wild type, 37 Tg2576, 32 FoxO1DN, and 11 FoxO1DNTg2576) and 97 male mice (38 wild type, 29 Tg2576, 21 FoxO1DN, and 9 FoxO1DNTg2576). Insulin tolerance tests (ITT) of mice aged 10–11 weeks. ITT was assessed from 116 female (38 wild type, 36 Tg2576, 32 FoxO1DN, and 10

FoxO1DNTg2576) and 98 male mice (38 wild type, 29 Tg2576, 22 FoxO1DN, and 9 FoxO1DNTg2576). **c** Western blot analysis of FoxO1DN and eGFP expression in hippocampus and cortex of FoxO1DN mice and respective controls. **d** Western blot of hippocampal expression of amyloid precursor protein (APP), Western blot analysis of C-terminal fragment- α (α CTF), and β CTF at 60 weeks of age. **e** Western blot analysis of A β oligomers using β -amyloid_{1–17} antibodies at 60 weeks of age. **f** ELISA of β -amyloid_{1–40/1–42} peptides from A β -guanidine-HCl extractions of hippocampi at 60 weeks of age. $n = 4$ per group. Data represent means $\pm$ SEM. $*p < 0.05$ (Student's t test)

proteotoxicity is at least partially responsible for the premature lethality in case of APP overexpression (El Khoury et al. 2007; Greeve et al. 2004; Leissring et al. 2003; Matsubara et al. 2003; Meilandt et al. 2009; Nathan et al. 2005; Rajendran et al. 2008). In humans, the APP gene is located on chromosome 21 and APP is overexpressed in patients with Down's syndrome (trisomy 21). Accordingly, Down's syndrome is associated with increased mortality (Baird and Sadovnick 1988;

Yang et al. 2002) even compared with people with other mental retardations (Day et al. 2005; Hermon et al. 2001; Strauss and Eyman 1996). In older age, AD is one of the leading causes of death in trisomy 21. Even isolated duplication of the APP gene causes AD and is associated with premature death (Cabrejo et al. 2006; Rovelet-Lecrux et al. 2006; Sleegers et al. 2006).

The DAF-2 signaling pathway in *C. elegans* (ortholog to the mammalian IR/IGF-1Rs) has been

identified as possible modulator of A β proteotoxicity without altering total A β burden. Cohen et al. have shown that knocking down DAF-2 in *C. elegans* expressing human A β _{1–42} reduces A β toxicity (Cohen et al. 2006). This effect was mediated by DAF-16, and HSF-1. DAF-16 mediates the formation of low toxic, high molecular weight aggregates from highly toxic small molecular weight aggregates (A β hyperaggregation) (Cohen et al. 2006). Recently, A β hyperaggregation has been identified as mechanism of A β detoxification in an IGF-1 resistant mouse model of AD (Cohen et al. 2009). In this study, IGF-1R^{+/-} mice were crossed with mice expressing the Swedish mutant of APP and the Δ E9 variant of presenilin 1. These mice were protected from cognitive decline and neurodegeneration usually observed in this Alzheimer model suggesting that partial IGF-1 resistance decreases A β toxicity possibly in combination with increased cellular stress resistance in response to A β (Cohen et al. 2009; Douglas and Dillin 2010; Killick et al. 2009). In addition, neuron-specific knockout of both copies of the IGF-1R gene rescues premature mortality and decreases A β load in Tg2576 mice (Freude et al. 2009).

Here, we show that neuron-specific deletion of only one copy of the IGF-1R reduces premature mortality of Tg2576 mice and decreases the concentration of A β oligomers without altering total A β load or APP expression. These data reinforce the role of IGF-1R signaling in the context of proteotoxicity suggesting IGF-1R signaling represents a regulatory pathway of protein turnover, and aggregation as suggested by previous studies (Cohen et al. 2006, 2009).

In mammals, the IIS is divided into the IR and IGF-1R signaling pathway. To specifically define the role of IRs in this context, we generated neuron-specific IR knockout mice and crossed them with Tg2576 mice. Surprisingly, we found that in contrast to complete deletion of neuronal IGF-1R (Freude et al. 2009), IR deficiency does not protect Tg2576 mice from premature death. Interestingly, A β _{1–40} and A β _{1–42} concentrations were significantly lower in nIR^{-/-}Tg2576 compared with Tg2576 mice but not in nIGF-1R^{+/-}Tg2576 mice. These data might suggest that total A β concentrations are not responsible for the previously described rescue of the premature mortality mediated via reduced expression of the IGF-1Rs. This in turn indicates that quality of A β aggregates might be specifically

influenced by IGF-1R but not IR. Recent data suggest that concentrations of oligomeric A β are the most important cytotoxic A β species, we therefore investigated concentrations of oligomeric A β in our mutant mice. Interestingly, we observed a reduction of oligomeric A β in nIGF-1R^{+/-}Tg2576 and nIR^{-/-}Tg2576 mice compared with Tg2576 mice excluding that concentrations of oligomeric A β alone are responsible for the difference between the effect of IGF-1R- and IR-mediated signals on premature death of Tg2576 mice. Our data support a hypothesis that IGF-mediated signals does not only reduce oligomeric A β concentration and toxicity as previously shown (Cohen et al. 2006, 2010, 2009) but also influences cellular response to proteotoxicity via so far unknown mechanisms.

Interestingly, both deletion of the IR or the IGF-1R decreases APP^{sw} processing in vivo. In order to further analyze the role of IIS signaling for endogenous APP processing, we generated IRS-2 knockdown cells. Previous work in SHSY5Y cells as well as in primary cultured neurons suggested that chronic treatment with IGF-1 induces a shift from TrkA to p75^{NTR} expression as seen in aging brains (Costantini et al. 2006). This switch might increase β -secretase activity indirectly by activation of neuronal sphingomyelinase which is responsible for the liberation of ceramides acting as second messenger (Puglielli 2008) leading to stabilization of BACE-1 (Puglielli et al. 2003). This process has been proposed to be responsible for IGF-1's effect on A β generation. However, using SHSY5Y cells, we could show that IRS-2 knockdown influences α - and β -secretase activity via PI3-kinase pathway independent of secretase expression suggesting that IRS-2-mediated signals act on multiple levels on endogenous APP processing. Since IRS-2 might mediate not only insulin's or IGF-1's intracellular effects, we analyzed the impact of neuronal IRs and IGF-1Rs signaling on β -secretase activity in vivo. According to our in vitro experiments β -secretase activity was decreased in hippocamal lysates isolated from nIR^{-/-} and IGF-1R^{-/-} mice. This might explain the decreased A β burden observed in nIGF-1R^{-/-}Tg2576 (Freude et al. 2009) and the nIR^{-/-}Tg2576 mice reported here. However, as proven by the analysis of the nIGF-1R^{+/-}Tg2576 mice, total A β burden alone does not explain the survival differences observed between nIGF-1R^{-/-}Tg2576 or nIGF-1R^{+/-}Tg2576 and nIR^{-/-}Tg2576 mice.

In order to gain further insights into the molecular mechanism how IGF-1 signaling influences survival and APP processing *in vivo*, we took advantage of the observation in *C. elegans* that A β hyperaggregation and detoxification depends of DAF-16 target genes (Cohen et al. 2006). The mammalian orthologs to DAF-16 are the FoxO transcription factors.

FoxO transcription factors act as downstream target of the insulin/IGF-1 cascade, which down-regulates FoxO activity via Akt-mediated phosphorylation, in turn triggering nuclear exclusion and degradation of the FoxO transcription factors. There are four different members of the mammalian FoxO-family: FoxO1, FoxO3a, FoxO4, and FoxO6. Apart from FoxO6, which is exclusively expressed in the brain, and FoxO4, which has not been found in brain (Hoekman et al. 2006; Jacobs et al. 2003), the other FoxO transcription factors are found ubiquitously. Because of the shared DNA-binding domain, FoxOs are expected to bind to similar DNA sequences (Furuyama et al. 2000). Thus, all FoxOs might be able to regulate the same set of genes through binding to this sequence. Indeed, large overlap in gene expression is observed when comparing the transcriptional activity during overexpression of the individual FoxOs. Functional specificity is most likely achieved via interaction with co-regulators (Burgering 2008). Since there are three different FoxOs generally expressed in rodent brain, we investigated the expression pattern of the three FoxOs in different brain regions. Interestingly, there are region-specific differences in FoxO expression. In the hippocampus, FoxO1 expression is nearly twice as high compared with FoxO3a, and even five times higher than FoxO6. However, in the frontal and parietal cortex, FoxO3a is significantly higher expressed in comparison to FoxO1 and FoxO6. Since the Cre transgenic mice used in the present study for all genetic manipulations expresses predominantly in the hippocampus, we asked the question whether FoxO1 has any influence of A β burden and survival. Therefore, we created mice expressing a dominant negative (FoxO1DN) and a constitutively active FoxO1 (FoxO1ADA). Our approach using the FoxO1DN and ADA mutants to inhibit or activate FoxO1-mediated transcription has been successfully used in previous studies and is well characterized (Adachi et al. 2007; Belgardt et al. 2008; Kitamura et al. 2007). Analysis of the FoxO1DN and FoxO1ADA in a Tg2576 background

showed that activation or inhibition of FoxO1-mediated transcription does not explain the phenotypes of nIR^{-/-}Tg2576, nIGF-1R^{-/-}Tg2576, or nIGF-1^{+/-}Tg2576 mice. Surprisingly, expression of FoxO1ADA in Tg2576 mice led to a so far unexplained excessive mortality. Thus, another pathway or one of the other FoxO transcription factors mediates the protective effect on overall survival observed in Tg2576 mice harboring either haploinsufficiency or homozygous deletion of the IGF-1R.

In summary, we have shown that (1) neuron-specific deletion of both copies of the IR and the IGF-1R (Freude et al. 2009) reduced APP processing and A β burden. (2) IR and IGF-1 receptor signaling regulates endosomal APP processing via PI3K dependent pathway. (3) Decreased IGF-1R but not IR expression reduces premature mortality in Tg2576 mice via a so far unknown mechanism not dependent on total A β load or the concentrations of soluble A β oligomers. (4) Neuronal FoxO1-mediated transcription does not explain the phenotypes observed either in nIR^{-/-}Tg2576 or nIGF-1^{+/-}Tg2576 mice.

Acknowledgments We thank Prof. Dr. R. Wiesner for critical discussion of the data and the manuscript. This work was supported by the Alzheimer Forschungsinitiative e.V. (to MS # 08813) and the Else-Kröner-Fresenius Stiftung (to MS #2010_A93) and Köln Fortune (to MS and KS)

References

- Adachi M, Osawa Y, Uchinami H, Kitamura T, Accili D, Brenner DA (2007) The forkhead transcription factor FoxO1 regulates proliferation and transdifferentiation of hepatic stellate cells. *Gastroenterology* 132:1434–1446
- Adlerz L, Holback S, Multhaup G, Iverfeldt K (2007) IGF-1-induced processing of the amyloid precursor protein family is mediated by different signaling pathways. *J Biol Chem* 282:10203–10209
- Baird PA, Sadovnick AD (1988) Life expectancy in Down syndrome adults. *Lancet* 2:1354–1356
- Belgardt BF, Husch A, Rother E, Ernst MB, Wunderlich FT, Hampel B, Klockener T, Alessi D, Kloppenburg P, Bruning JC (2008) PDK1 deficiency in POMC-expressing cells reveals FoxO1-dependent and -independent pathways in control of energy homeostasis and stress response. *Cell Metab* 7:291–301
- Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Muller-Wieland D, Kahn CR (2000) Role of brain insulin receptor in control of body weight and reproduction. *Science* 289:2122–2125
- Burgering BM (2008) A brief introduction to FOXology. *Oncogene* 27:2258–2262

- Cabrejo L, Guyant-Marechal L, Laquerriere A, Vercelletto M, De la FF, Thomas-Anterion C, Verny C, Letournel F, Pasquier F, Vital A, Checler F, Frebourg T, Campion D, Hannequin D (2006) Phenotype associated with APP duplication in five families. *Brain* 129:2966–2976
- Carlson GA, Borchelt DR, Dake A, Turner S, Danielson V, Coffin JD, Eckman C, Meiners J, Nilsen SP, Younkin SG, Hsiao KK (1997) Genetic modification of the phenotypes produced by amyloid precursor protein overexpression in transgenic mice. *Hum Mol Genet* 6:1951–1959
- Chishti MA, Yang DS, Janus C, Phinney AL, Horne P, Pearson J, Strome R, Zuker N, Loukides J, French J, Turner S, Lozza G, Grilli M, Kunicki S, Morissette C, Paquette J, Gervais F, Bergeron C, Fraser PE, Carlson GA, George-Hyslop PS, Westaway D (2001) Early-onset amyloid deposition and cognitive deficits in transgenic mice expressing a double mutant form of amyloid precursor protein 695. *J Biol Chem* 276:21562–21570
- Cohen E, Bieschke J, Perciavalle RM, Kelly JW, Dillin A (2006) Opposing activities protect against age-onset proteotoxicity. *Science* 313:1604–1610
- Cohen E, Paulsson JF, Blinder P, Burstyn-Cohen T, Du D, Estepa G, Adame A, Pham HM, Holzenberger M, Kelly JW, Masliah E, Dillin A (2009) Reduced IGF-1 signaling delays age-associated proteotoxicity in mice. *Cell* 139:1157–1169
- Cohen E, Du D, Joyce D, Kapernick EA, Volovik Y, Kelly JW, Dillin A (2010) Temporal requirements of insulin/IGF-1 signaling for proteotoxicity protection. *Aging Cell* 9:126–134
- Costantini C, Scrable H, Puglielli L (2006) An aging pathway controls the TrkA to p75NTR receptor switch and amyloid beta-peptide generation. *EMBO J* 25:1997–2006
- Craft S (2009) The role of metabolic disorders in Alzheimer disease and vascular dementia: two roads converged. *Arch Neurol* 66:300–305
- Crews L, Masliah E (2010) Molecular mechanisms of neurodegeneration in Alzheimer's disease. *Hum Mol Genet* 19: R12–R20
- Day SM, Strauss DJ, Shavelle RM, Reynolds RJ (2005) Mortality and causes of death in persons with Down syndrome in California. *Dev Med Child Neurol* 47:171–176
- de la Monte SM, Tong M, Lester-Coll N, Plater M Jr, Wands JR (2006) Therapeutic rescue of neurodegeneration in experimental type 3 diabetes: relevance to Alzheimer's disease. *J Alzheimers Dis* 10:89–109
- Douglas PM, Dillin A (2010) Protein homeostasis and aging in neurodegeneration. *J Cell Biol* 190:719–729
- El Khoury J, Toft M, Hickman SE, Means TK, Terada K, Geula C, Luster AD (2007) Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. *Nat Med* 13:432–438
- Finch CE, Ruvkun G (2001) The genetics of aging. *Annu Rev Genomics Hum Genet* 2:435–462
- Freude S, Hettich MM, Schumann C, Stohr O, Koch L, Kohler C, Udelhoven M, Leeser U, Muller M, Kubota N, Kadowaki T, Krone W, Schroder H, Bruning JC, Schubert M (2009) Neuronal IGF-1 resistance reduces A β accumulation and protects against premature death in a model of Alzheimer's disease. *FASEB J* 23:3315–3324
- Frolich L, Blum-Degen D, Bernstein HG, Engelsberger S, Humrich J, Laufer S, Muschner D, Thalheimer A, Turk A, Hoyer S, Zochling R, Boissl KW, Jellinger K, Riederer P (1998) Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. *J Neural Transm* 105:423–438
- Frolich L, Blum-Degen D, Riederer P, Hoyer S (1999) A disturbance in the neuronal insulin receptor signal transduction in sporadic Alzheimer's disease. *Ann N Y Acad Sci* 893:290–293
- Furuyama T, Nakazawa T, Nakano I, Mori N (2000) Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* 349:629–634
- Greeve I, Kretschmar D, Tschape JA, Beyn A, Brellinger C, Schweizer M, Nitsch RM, Reifegerste R (2004) Age-dependent neurodegeneration and Alzheimer-amyloid plaque formation in transgenic *Drosophila*. *J Neurosci* 24:3899–3906
- Hermon C, Alberman E, Beral V, Swerdlow AJ (2001) Mortality and cancer incidence in persons with Down's syndrome, their parents and siblings. *Ann Hum Genet* 65:167–176
- Ho L, Qin W, Pompl PN, Xiang Z, Wang J, Zhao Z, Peng Y, Cambareri G, Rocher A, Mobbs CV, Hof PR, Pasinetti GM (2004) Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. *FASEB J* 18:902–904
- Hoekman MF, Jacobs FM, Smidt MP, Burbach JP (2006) Spatial and temporal expression of FoxO transcription factors in the developing and adult murine brain. *Gene Expr Patterns* 6:134–140
- Hsiao KK, Borchelt DR, Olson K, Johannsdottir R, Kitt C, Yunis W, Xu S, Eckman C, Younkin S, Price D (1995) Age-related CNS disorder and early death in transgenic FVB/N mice overexpressing Alzheimer amyloid precursor proteins. *Neuron* 15:1203–1218
- Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G (1996) Correlative memory deficits, A β elevation, and amyloid plaques in transgenic mice. *Science* 274:99–102
- Isacson O, Seo H, Lin L, Albeck D, Granholm AC (2002) Alzheimer's disease and Down's syndrome: roles of APP, trophic factors and ACh. *Trends Neurosci* 25:79–84
- Jacobs FM, van der Heide LP, Wijchers PJ, Burbach JP, Hoekman MF, Smidt MP (2003) FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. *J Biol Chem* 278:35959–35967
- Jacobsen KT, Adler L, Multhaup G, Iverfeldt K (2010) Insulin-like growth factor-1 (IGF-1)-induced processing of amyloid-beta precursor protein (APP) and APP-like protein 2 is mediated by different metalloproteinases. *J Biol Chem* 285:10223–10231
- Kappeler L, Filho CM, Dupont J, Leneuve P, Cervera P, Perin L, Loudes C, Blaise A, Klein R, Epelbaum J, Le Bouc Y, Holzenberger M (2008) Brain IGF-1 receptors control mammalian growth and lifespan through a neuroendocrine mechanism. *PLoS Biol* 6:e254
- Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A *C. elegans* mutant that lives twice as long as wild type. *Nature* 366:461–464

- Killick R, Scales G, Leroy K, Causevic M, Hooper C, Irvine EE, Choudhury AI, Drinkwater L, Kerr F, Al Qassab H, Stephenson J, Yilmaz Z, Giese KP, Brion JP, Withers DJ, Lovestone S (2009) Deletion of *Irs2* reduces amyloid deposition and rescues behavioural deficits in APP transgenic mice. *Biochem Biophys Res Commun* 386:257–262
- Kitamura T, Kitamura YI, Funahashi Y, Shawber CJ, Castrillon DH, Kollipara R, DePinho RA, Kitajewski J, Accili D (2007) A Foxo/Notch pathway controls myogenic differentiation and fiber type specification. *J Clin Invest* 117:2477–2485
- Kroner Z (2009) The relationship between Alzheimer's disease and diabetes: type 3 diabetes? *Altern Med Rev* 14:373–379
- Lee RY, Hench J, Ruvkun G (2001) Regulation of *C. elegans* DAF-16 and its human ortholog FKHRL1 by the daf-2 insulin-like signaling pathway. *Curr Biol* 11:1950–1957
- Leissring MA, Farris W, Chang AY, Walsh DM, Wu X, Sun X, Frosch MP, Selkoe DJ (2003) Enhanced proteolysis of beta-amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death. *Neuron* 40:1087–1093
- Lesne S, Koh MT, Kotilinek L, Kaye R, Glabe CG, Yang A, Gallagher M, Ashe KH (2006) A specific amyloid-beta protein assembly in the brain impairs memory. *Nature* 440:352–357
- Lin K, Dorman JB, Rodan A, Kenyon C (1997) daf-16: An HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. *Science* 278:1319–1322
- Lovestone S (1999) Diabetes and dementia: is the brain another site of end-organ damage? *Neurology* 53:1907–1909
- Marks N, Berg MJ (2010) BACE and gamma-secretase characterization and their sorting as therapeutic targets to reduce amyloidogenesis. *Neurochem Res* 35:181–210
- Matsubara E, Bryant-Thomas T, Pacheco QJ, Henry TL, Poeggeler B, Herbert D, Cruz-Sanchez F, Chyan YJ, Smith MA, Perry G, Shoji M, Abe K, Leone A, Grundke-Iqbal I, Wilson GL, Ghiso J, Williams C, Refolo LM, Pappolla MA, Chain DG, Neria E (2003) Melatonin increases survival and inhibits oxidative and amyloid pathology in a transgenic model of Alzheimer's disease. *J Neurochem* 85:1101–1108
- Meilandt WJ, Cisse M, Ho K, Wu T, Esposito LA, Searce-Levie K, Cheng IH, Yu GQ, Mucke L (2009) Neprilysin overexpression inhibits plaque formation but fails to reduce pathogenic A β oligomers and associated cognitive deficits in human amyloid precursor protein transgenic mice. *J Neurosci* 29:1977–1986
- Moechars D, Dewachter I, Lorent K, Reverse D, Baekelandt V, Naidu A, Tessier I, Spittaels K, Haute CV, Checler F, Godaux E, Cordell B, Van Leuven F (1999) Early phenotypic changes in transgenic mice that overexpress different mutants of amyloid precursor protein in brain. *J Biol Chem* 274:6483–6492
- Moloney AM, Griffin RJ, Timmons S, O'Connor R, Ravid R, O'Neill C (2008) 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* 31(2):224–243
- Moloney AM, Griffin RJ, Timmons S, O'Connor R, Ravid R, O'Neill C (2010) 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* 31:224–243
- Nathan C, Calingasan N, Nezezon J, Ding A, Lucia MS, La Perle K, Fuortes M, Lin M, Ehrt S, Kwon NS, Chen J, Vodovotz Y, Kipiani K, Beal MF (2005) Protection from Alzheimer's-like disease in the mouse by genetic ablation of inducible nitric oxide synthase. *J Exp Med* 202:1163–1169
- Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, Ruvkun G (1997) The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature* 389:994–999
- Ott A, Stolk RP, Hofman A, van Harskamp F, Grobbee DE, Breteler MM (1996) Association of diabetes mellitus and dementia: the Rotterdam Study. *Diabetologia* 39:1392–1397
- Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM (1999) Diabetes mellitus and the risk of dementia: the Rotterdam Study. *Neurology* 53:1937–1942
- Pilcher H (2006) Alzheimer's disease could be "type 3 diabetes". *Lancet Neurol* 5:388–389
- Pugliese L (2008) Aging of the brain, neurotrophin signaling, and Alzheimer's disease: is IGF1-R the common culprit? *Neurobiol Aging* 29:795–811
- Pugliese L, Ellis BC, Saunders AJ, Kovacs DM (2003) Ceramide stabilizes beta-site amyloid precursor protein-cleaving enzyme 1 and promotes amyloid beta-peptide biogenesis. *J Biol Chem* 278:19777–19783
- Rajendran L, Schneider A, Schlechtingen G, Weidlich S, Ries J, Braxmeier T, Schulle P, Schulz JB, Schroeder C, Simons M, Jennings G, Knolker HJ, Simons K (2008) Efficient inhibition of the Alzheimer's disease beta-secretase by membrane targeting. *Science* 320:520–523
- Rivera EJ, Goldin A, Fulmer N, Tavares R, Wands JR, de la Monte SM (2005) 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* 8:247–268
- Rovelet-Lecrux A, Hannequin D, Raux G, Le Meur N, Laquerriere A, Vital A, Dumanchin C, Feuillette S, Brice A, Vercelletto M, Dubas F, Frebourg T, Campion D (2006) APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. *Nat Genet* 38:24–26
- Sleegers K, Brouwers N, Gijssels I, Theuns J, Goossens D, Wauters J, Del Favero J, Cruts M, van Duijn CM, Van Broeckhoven C (2006) APP duplication is sufficient to cause early onset Alzheimer's dementia with cerebral amyloid angiopathy. *Brain* 129:2977–2983
- Stachelscheid H, Ibrahim H, Koch L, Schmitz A, Tschamtk M, Wunderlich FT, Scott J, Michels C, Wickenhauser C, Haase I, Bruning JC, Niessen CM (2008) Epidermal insulin/IGF-1 signalling control interfollicular morphogenesis and proliferative potential through Rac activation. *EMBO J* 27:2091–2101
- Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM (2005) Impaired insulin and insulin-like growth factor expression and

- signaling mechanisms in Alzheimer's disease—is this type 3 diabetes? *J Alzheimers Dis* 7:63–80
- Stein TD, Johnson JA (2002) Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. *J Neurosci* 22:7380–7388
- Strauss D, Eyman RK (1996) Mortality of people with mental retardation in California with and without Down syndrome, 1986–1991. *Am J Ment Retard* 100:643–653
- Taguchi A, Wartschow LM, White MF (2007) Brain IRS2 signaling coordinates life span and nutrient homeostasis. *Science* 317:369–372
- Willuweit A, Velden J, Godemann R, Manook A, Jetzek F, Tintrup H, Kauselmann G, Zevnik B, Henriksen G, Drzezga A, Pohlner J, Schoor M, Kemp JA, von der Kammer K (2009) Early-onset and robust amyloid pathology in a new homozygous mouse model of Alzheimer's disease. *PLoS One* 4:e7931
- Wisniewski KE, Wisniewski HM, Wen GY (1985) Occurrence of neuropathological changes and dementia of Alzheimer's disease in Down's syndrome. *Ann Neurol* 17:278–282
- Wolkow CA, Kimura KD, Lee MS, Ruvkun G (2000) Regulation of *C. elegans* life-span by insulinlike signaling in the nervous system. *Science* 290:147–150
- Yang Q, Rasmussen SA, Friedman JM (2002) Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. *Lancet* 359:1019–1025