Differential involvement of insulin receptor substrate (IRS)-1 and IRS-2 in brain insulin signaling is associated with the effects on amyloid pathology in a mouse model of Alzheimer’s disease

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ABSTRACT

Insulin signaling has been implicated in the metabolism as well as aging and longevity. Type 2 diabetes mellitus and its core pathology, insulin resistance, has also been implicated in the development of Alzheimer’s disease (AD) and amyloid-β deposition in humans. By contrast, genetic ablation of the insulin/IGF-1 signaling (IIS) pathway components, e.g. insulin receptor substrate (IRS)-2, has been documented to suppress amyloid-β accumulation in the brains of transgenic mice overexpressing AD mutant β-amyloid precursor protein (APP). Therefore, the brain IIS may be a key modifiable molecular target in the pathophysiology of AD. IRS-1 and IRS-2 are critical nodes in IIS as substrates for insulin receptor and IGF-1 receptor, although the functional differences between IRS-1 and IRS-2 in the adult brain are yet to be explored. To examine their relative contribution to the brain IIS activity and AD pathomechanism, we generated APP transgenic mice lacking either IRS-1 or IRS-2. IRS-1 deficiency had little effects on the brain IIS pathway associated with compensatory activation of IRS-2, whereas IRS-2 deficiency was not fully compensated by activation of IRS-1, and the downstream activation of Akt also was significantly compromised. Pathological analyses of the cortical tissues showed that the biochemical levels of soluble and insoluble amyloid-β, the amyloid-β histopathology, and tau phosphorylation were not affected by the absence of IRS-1, in contrast to the marked alteration in IRS-2 deleted mice. These results suggest the predominance of IRS-2 in the brain IIS, and support the hypothesis that reduced IIS exerts anti-amyloid effects in the brain.

1. Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder causing cognitive decline and dementia. AD is pathologically characterized by the extracellular deposition of senile plaques composed of amyloid-β peptides (Aβ), intracellular neurofibrillary tangles consisting of abnormally hyperphosphorylated tau, and the loss of cerebrcortical neurons in the brain. Several lines of evidence suggest the pathogenetic significance of Aβ in the neurodegenerative process of AD: Aβ deposition is the earliest change in the brains of patients with AD, and most importantly, mutations linked to autosomal-dominantly inherited familial AD in the APP, PSEN1 and PSEN2 genes altogether have effects to

Abbreviations: Alzheimer’s disease, AD; amyloid-β peptide, Aβ; insulin receptor, IR; insulin receptor substrate, IRS; insulin/insulin-like growth factor-1, IGF-1; insulin/insulin-like growth factor-1 signaling, IIS; phosphoinositide 3-kinase, PI3K; type 2 diabetes mellitus, T2DM.

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promote deposition of Aβ, supporting the causative role of Aβ in the pathophysiology of AD (Selkoe and Hardy, 2016).

Recently, a relationship between the pathogenesis of AD and insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) has been highlighted. The IIS pathway plays multiple roles in regulating basic cellular functions such as growth, survival, and metabolism in a variety of organisms (Saltiel and Kahn, 2001; Taguchi and White, 2008). In studies using mouse models of AD that develop amyloid plaques in the brain by transgenic overexpression of familial AD mutant APP, genetic inhibition of insulin receptor substrate-2 (IRS-2) suppressed Aβ accumulation and improved behavioral deficits (Freude et al., 2009; Killlick et al., 2009; Wakabayashi et al., 2019). Similar effects have been reported in AD model mice that lack IRS receptors, i.e. the insulin receptor (IR) and IGF-1 receptor (IGF-1R), specifically in neurons (Freude et al., 2009; Gontier et al., 2015; Söhr et al., 2013). On the other hand, epidemiological studies have shown that the incidence of AD is about 1.5 times higher in patients with type 2 diabetes mellitus (T2DM) (Bisselés et al., 2006; Gudala et al., 2013). It has also been suggested that insulin resistance is associated with exacerbation of AD pathology (Matsuzaki et al., 2010). Furthermore, IRS-2 has been shown to be disturbed in the brains of patients with AD (De Felice and Ferreira, 2014; Steen et al., 2005; Talbot et al., 2012). In autopsied AD brains, serine-phosphorylated IRS-1, an indicator of insulin resistance in peripheral organs, was increased and correlated positively with Aβ plaque load and negatively with episodic and working memories (Talbot et al., 2012). These observations suggest that impaired IIS, i.e. insulin resistance, might represent a common exacerbating factor in T2DM and AD. Based on these findings, intranasal administration of insulin, adopted in an attempt to activate insulin signaling in the brain, was shown to improve cognitive function in humans with or without AD (Benedict et al., 2004; Reger et al., 2008).

However, we have recently found that high-fat diet feeding in IRS-2 deficient APP transgenic mice abolished the anti-amyloid effect and increased Aβ deposition. This suggests that upstream factors associated with the metabolic load, rather than impaired IRS per se, are likely to be involved in the acceleration of amyloid pathology (Ochiai et al., 2021; Wakabayashi et al., 2019). These observations may support an alternative view that disturbed IRS in AD brains might be a consequence or compensatory reactions of AD changes, and it may even be possible that native view that disturbed IIS in AD brains might be a consequence or exacerbating factor in T2DM and AD. Based on these findings, intranasal administration of insulin, adopted in an attempt to activate insulin signaling in the brain, was shown to improve cognitive function in humans with or without AD (Benedict et al., 2004; Reger et al., 2008).

In the canonical IIS pathway, activated IR and IGF-1R mainly transduce two distinct signaling pathways, the phosphoinositide 3-kinase (PI3K)-Akt and mitogen-activated protein kinase-extracellular signal-regulated kinase (MAPK-Erk) pathways, via tyrosine phosphorylation of IRS family proteins (Taniguchi et al., 2006). IRS-1 and IRS-2 are the widely expressed, major substrates of IR/IGF-1R in the IIS activation of recruited IRS family proteins (Taniguchi et al., 2006). IRS-1 and IRS-2 play overlapping and distinct functions: systemic insulin resistance and impaired IIS acts against the amyloid pathology (Stanley et al., 2016b).

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In the brain, IRS-2 has been established as an important regulator of tissue growth, neuronal survival, synaptic transmission, and glial metabolism, and is involved in the modulation of learning and memory, energy homeostasis, and feeding behavior (Fernandez and Torres-Alemán, 2012; Kleinridders et al., 2014). Importantly, however, the relative contribution of IRS-1 and IRS-2 to the IIS activity in the adult brain remains to be fully explored. Furthermore, it remains unknown whether the IIS activity dependent on IRS-1 is mechanistically involved in the AD pathophysiology. In this study, we generated APP transgenic mice (A7-Tg mice) deficient in IRS-1 or IRS-2 and examined their roles in the brain IRS. We also studied the AD-related pathological phenotypes in the brains of IRS-1-deficient A7-Tg mice, to determine the relationship between the activity of IIS and AD pathophysiology.

2. Experimental procedures

2.1. Mice

A7 transgenic mice (A7-Tg) overexpressing human APP695 harboring KM670/671NL and T714I familial AD mutations under the control of the Thy-1.2 promoter (Yamada et al., 2009) were backcrossed and maintained on a C57BL/6J background. IRS1−/− and IRS2−/− mice were generated as previously described (Kubota et al., 2000; Tamemoto et al., 1994). IRS1−/− and IRS2−/− mice were crossed with A7-Tg mice to generate IRS1−/−;A7-Tg mice and IRS2−/−;A7-Tg mice, and littermate controls were used. The animal care and experimental procedures were approved by the animal experiment committee of The University of Tokyo Graduate School of Medicine (approval no. P18-102). Mice were maintained on a 12 h light-dark cycle and provided ad libitum access to water and a standard chow. The genders of the mice used in the experiments are indicated in the corresponding figure legends.

2.2. Antibodies

The following antibodies were used: anti-IR (06-248, Merck), anti-α-tubulin (DM1A, Merck), anti-phosphotyrosine (clone 4G10, 05-321, Merck), anti-phospho-IRS-1 (Ser307) (2381, Cell Signaling Technology (CST)), anti-IRS-2 (sc-8299, Santa Cruz Biotechnology), anti-IRS-2 (sc-390761, Santa Cruz Biotechnology), anti-phospho-Akt (Ser473) (9271, CST), anti-phospho-Akt (Thr308) (13083, CST), Akt (9272, CST), anti-IRβ (sc-711, Santa Cruz Biotechnology), anti-IGF-1Rβ (sc-713, Santa Cruz Biotechnology), anti-phospho-p70 S6 Kinase (Thr389) (9205, CST), anti-p70 S6 Kinase (9202, CST), Tau (Tau-5, AHB0042, Thermo Fisher), anti-phospho-Tau (Ser202, Thermo Fisher), anti-phospho-Tau (Thr212, 82E1, IBL), peroxidase-conjugated AffiniPure anti-rabbit IgG (110-035-003, Jackson ImmunoResearch), and peroxidase-conjugated AffiniPure anti-mouse IgG (515-035-003, Jackson ImmunoResearch).

2.3. Isolation of different brain cell populations

Preparation of a single-cell suspension was done based on earlier protocols (Brewer and Torricelli, 2007; Orre et al., 2014). C57BL/6 J mice were anesthetized using combination anesthetic agents including 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol, and transcardially perfused with Hank’s Balanced Salt Solution without calcium or magnesium (HBSS) (Thermo Fisher). The following antibodies were used: anti-IRS1 (06-248, Merck), anti-α-tubulin (DM1A, Merck), anti-phosphotyrosine (clone 4G10, 05-321, Merck), anti-phospho-IRS-1 (Ser307) (2381, Cell Signaling Technology (CST)), anti-IRS-2 (sc-8299, Santa Cruz Biotechnology), anti-IRS-2 (sc-390761, Santa Cruz Biotechnology), anti-phospho-Akt (Ser473) (9271, CST), anti-phospho-Akt (Thr308) (13083, CST), Akt (9272, CST), anti-IRβ (sc-711, Santa Cruz Biotechnology), anti-IGF-1Rβ (sc-713, Santa Cruz Biotechnology), anti-phospho-p70 S6 Kinase (Thr389) (9205, CST), anti-p70 S6 Kinase (9202, CST), Tau (Tau-5, AHB0042, Thermo Fisher), anti-phospho-Tau (Ser202, Thermo Fisher), anti-phospho-Tau (Thr212, 82E1, IBL), peroxidase-conjugated AffiniPure anti-rabbit IgG (110-035-003, Jackson ImmunoResearch), and peroxidase-conjugated AffiniPure anti-mouse IgG (515-035-003, Jackson ImmunoResearch).
to obtain a single-cell suspension. After the removal of cell debris and erythrocytes using the Debris Removal Solution and Red Blood Cell Lysis Solution (Milenyi Biotec), neurons, astrocytes, and microglia were magnetically isolated using Neuron Isolation Kit, Anti-ACSA-2 MicroBead Kit, CD11b (Microglia) MicroBeads, and MACS Separator (Milenyi Biotec), respectively, according to the manufacturer’s instructions.

2.4. Quantitative reverse transcription PCR

Total RNA was isolated using TRIzol Plus RNA Purification Kit and PureLink RNA Mini kit (Thermo Fisher). RNA purity and concentration were measured by NanoDrop (ThermoFisher). Total RNA was reverse-transcribed into cDNA using ReverTra Ace qPCR Master Mix with gDNA Remover (TOYOBO). Real-time PCR was performed with LightCycler 480 system (Roche) using THUNDERBIRD SYBR qPCR Mix (TAKARA BIO INC.). Threshold cycle values were normalized to Actb (TAKARA BIO INC.) using THUNDERBIRD SYBR qPCR Mix (TAKARA BIO INC.). Total RNA was reverse-transcribed into cDNA using ReverTra Ace qPCR Master Mix with gDNA Remover (TOYOBO). Real-time PCR was performed with LightCycler 480 system (Roche) using THUNDERBIRD SYBR qPCR Mix (TAKARA BIO INC.). Threshold cycle values were normalized to Actb (TAKARA BIO INC.) using THUNDERBIRD SYBR qPCR Mix (TAKARA BIO INC.).

2.5. Protein extraction and Western blot analysis

Brains were harvested, dissected into the hippocampal formation (referred to as the hippocampus), which includes the hippocampus, dentate gyrus, and subiculum, and the cerebral cortex, which includes the neocortex, cingulate, and piriform cortex, snap frozen in liquid nitrogen and stored at −80°C. For analysis of soluble and insoluble Aβs, brain tissues were homogenized in a 1:10 (w/v) volume of Tris-buffered saline (TBS), centrifuged at 347,600 g for 20 min at 4°C and supernatants were saved as TBS-soluble fractions. Resulting pellets were homogenized in a 1:10 (w/v) volume of 2% Triton X-100 in TBS and centrifuged at 347,600 g for 20 min at 4°C. Pellets were then homogenized in a 1:10 (w/v) volume of 2% SDS in TBS, incubated for 30 min at 37°C and centrifuged at 347,600 g for 20 min at 4°C. SDS-insoluble pellets were dissolved in 70% formic acid using a sonicator (Branson), centrifuged at 347,600 g for 20 min at 4°C and supernatants were desiccated by Speed-Vac followed by resuspension in DMSO. For analysis of IIS related proteins, brain tissues were homogenized in a 1:10 (w/v) volume of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS in TBS), and centrifuged at 17,800 g for 30 min at 4°C. Protein concentration was determined with BCA protein assay kit (TAKARA BIO INC.). All the buffers contained cOmplete protease inhibitor and PhosSTOP phosphatase inhibitor cocktails (Merck). For detection of tyrosine-phosphorylated IRS-1, IRS-2, IR, and IGF-1R, RIPA soluble fractions were incubated with antibodies recognizing each protein overnight at 4°C. Protein G agarose beads (ThermoFisher) were added and incubated for 2 h at 4°C. The samples were washed three times with RIPA buffer and eluted with Laemmli sample buffer containing 2% 2-mercaptoethanol.

For immunoblotting, samples were separated by SDS-polyacrylamide gel electrophoresis under a reducing condition using a Tris-Glycine gel system, transferred to polyvinylidene difluoride membrane (Merck) and reacted with antibodies. The immunoblots were developed using ImmunoStar reagents (Wako) and SuperSignal (Thermo Fisher), and visualized by LAS-4000 mini (Fujifilm).

2.6. Insulin perfusion via reverse microdialysis

Guide cannula (BASI) implantation in the hippocampus (from bregma: anteroposterior −3.1 mm, mediolateral −2.5 mm, dorsoventral −1.2 mm at 12° angle) was performed as described previously (Stanley et al., 2016a). Insulin (Humulin-R, Lilly) was infused directly into the hippocampus at 400 nM in artificial cerebrospinal fluid (aCSF; 1.3 mM CaCl₂, 1.2 mM MgSO₄·7H₂O, 3.0 mM KCl, 0.4 mM KH₂PO₄, 25 mM NaHCO₃ and 122 mM NaCl) containing 0.15% BSA at 1 μl/min for 1 h using microdialysis probes with 30 kDa molecular weight cut-off membranes (BASI). Controls received regular aCSF containing 0.15% BSA. Mice were immediately killed after 1 h of treatment and the hippocampus around the probe collected for analysis of IIS. Hippocampal insulin levels in the RIPA soluble fractions were quantitated by Ultra Sensitive “PLUS” Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc.).

2.7. ELISA quantitation of Aβ

To measure the Aβ levels in ISF and brain soluble fractions, an equal volume of 1 M guanidine hydrochloride was added and incubated for 30 min at room temperature. Levels of Aβ were quantitated by BNT77/BA27 or BNT77/BC05 Human/Rat β Amyloid ELISA kit (Wako).

2.8. Immunohistochemical analysis and morphometry

Mouse brains were fixed with 4% paraformaldehyde in PBS for 24 h, dehydrated and embedded in paraffin. Serial sections were cut at 4-μm thickness. Deparaffinized sections were treated with microwave (700 W) in citrate buffer pH 6.0 for 20 min, followed by digestion with 100 μg/ml proteinase K (Worthington) in TBS for 6 min at 37°C. After blocking by incubation with 10% calf serum in TBS, the sections were incubated with an anti-Aβ antibody 82E1 (IBL) and then a biotinylated anti-mouse IgG antibody (Vector Laboratories), followed by visualization by avidin-biotin complex method (ABC elite, Vector Laboratories) using diaminobenzidine as chromogen. The percentage area covered by Aβ immunoreactivity in cerebral cortex, hippocampus, and piriform cortex was measured using Image J software (NIH) as previously described (Yamamoto et al., 2015).

2.9. Statistical analysis

Quantitative data were analyzed statistically by unpaired t-test for two-group data, or one-way ANOVA followed by Tukey’s multiple comparisons test for three- or four-group data using GraphPad Prism 7. In figures, all data are represented by mean ± SEM. Statistical significance is indicated by *p < 0.05, **p < 0.01, and ***p < 0.001.

3. Results

3.1. IRS-1 and IRS-2 showed similar cellular expression patterns in the cerebrum

It has been shown that IRS-1 and IRS-2 are widely expressed throughout the brain (Kleinridders et al., 2014), although the specific expression patterns of IRS-1 and -2 in different types of cells in mature brains remained unclear. To address this issue, we isolated neurons, astrocytes, and microglial cells from the dissociated cellular suspensions of the cerebral hemisphere (including cerebral cortices and hippocampi) of adult C57BL/6J mice using magnetic cell sorting. The purity of each cellular fraction was verified by quantitative RT-PCR analysis using the following cell-specific genetic markers: Slc12a5 and Nefm (neuronal markers), Aqp4 and Slc12a5 (astrocytic markers), and Cx3cr1 and Aif1 (microglial markers) (Supplementary Fig. S1). Irs1 mRNA expression was detected in astrocytes and neurons, while it was very low in microglia (Fig. 1A). Irs2 mRNA expression was detected in all three cell...
types and, like that of IRS1, showed a tendency to be higher in astrocytes and neurons (Fig. 1B). These results suggest that the cellular expression patterns of IRS-1 and IRS-2 in brains are comparable in the cerebrum.

3.2. Deletion of IRS-1 or IRS-2 caused a compensatory activation of the other IRS in the cerebral cortex

In this study, transgenic mice overexpress human APP harboring the Swedish and Austrian mutations under the control of the Thy-1.2 promoter (A7-Tg) were used as a model of AD (Yamada et al., 2009). A7-Tg mice develop progressive Aβ deposition in brains starting at ~12 months of age. Earlier studies have shown altered levels of IRS-1 or its serine phosphorylation in the brains of AD patients as well as of mouse models of AD (Bomfim et al., 2012; Moloney et al., 2010; Talbot et al., 2012). We thus investigated whether such alterations of IRS-1 also occurred in our model. Immunoblot analysis of RIPA-soluble cortical extracts from 14- to 16-month-old A7-Tg and littermate control mice showed no difference in the levels of total, Tyr-phosphorylated and Ser307-phosphorylated IRS-1 (Supplementary Fig. S2). Therefore, it appears that abnormalities in insulin signaling associated with IRS-1 have not occurred in our A7-Tg mouse model. A possible explanation for the difference in results between autopsy brains of AD patients and A7-Tg mice would be that the changes in IRS-1 may be triggered in the presence of more advanced AD pathology, such as tau accumulation or severe neuroinflammation (Yarchon et al., 2014).

To gain insights into the significance of IRS signaling in the pathophysiology of AD, we generated AD model mice genetically lacking IRS-1 or IRS-2. A7-Tg mice used in this study express human APP harboring the Swedish and Austrian familial Alzheimer mutations under the control of the Thy-1.2 promoter (Yamada et al., 2009). IR and IGF-1R are activated upon binding of the ligands and phosphorylate tyrosine residues in IRS-1 and IRS-2, thereby transmitting the IIS pathways. We first analyzed the effect of deficiency of IRS-1 or IRS-2 on the activation of the rest of the IRS (IRS-2 or IRS-1, respectively) in the brain. In the cerebral cortices of IRS-1-deficient A7-Tg mice, the levels of tyrosine-phosphorylated IRS-2 were significantly increased by ~19% (Fig. 2A). The levels of tyrosine-phosphorylated IRS-1 also were significantly increased in the cortices of IRS-2-deficient A7-Tg mice, which was more marked compared with the compensatory increase of phosphorylated IRS-2 upon IRS-1 deficiency, by ~125% (Fig. 2B). Deletion of IRS-1 or IRS-2 had no effect on the basal levels of the IRS-2 and IRS-1 proteins, respectively (Fig. 2C and D). This reciprocal interaction between IRS-1 and IRS-2 was similarly observed in the cerebral cortex of mice without APP overexpression (Supplementary Fig. S3). These results suggest the occurrence of a compensatory activation of IRS-1 and IRS-2 by the deletion of the other type of IRS in the cerebral cortex.

3.3. Deletion of IRS-2, but not of IRS-1, reduced the basal activation of Akt

Tyrosine-phosphorylated IRS-1 and IRS-2 trigger the activation of PI3K, followed by generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) lipids. Akt/PKB binds to PIP3 at the cell membrane, thereby allowing partial activation of Akt by phosphorylation at Thr308 by PDK1. To achieve a full activation, Akt needs to be additionally phosphorylated at Ser473 by kinases such as mTORC2 (Alessi et al., 1996; Liu et al., 2014). The effect of IRS-2 deletion on Akt activation in the brain has not been consistently demonstrated; contradictory results have been observed, with reports of reduced phosphorylation of Akt Ser473 and GSK-3β, a direct substrate of Akt, while increased phosphorylation of GSK-3α (Costello et al., 2012; Freude et al., 2009; Killick et al., 2009; Schubert et al., 2003). It is also unclear how Akt phosphorylation in the brain is affected by the loss of IRS-1. We thus investigated the basal activation levels of Akt phosphorylation at Ser473 and Thr308 in the cerebral cortices of A7-Tg mice deficient in IRS-1 or IRS-2. IRS-1 deletion significantly increased Thr308 phosphorylation by ~71%, whereas Ser473 phosphorylation showed a slight trend of increase by ~14% (Fig. 3A–C). In contrast, IRS-2 deficiency reduced phosphorylation of Ser473 by ~11%, and phosphorylation of Thr308 also showed a trend of decrease in the cerebral cortex (Fig. 3E–G). Protein levels of Akt in the cortices of IRS-1 or IRS-2-deficient mice were unaltered compared with those of the control A7 mice (Fig. 3D and H). These results indicate that the basal activity of the cerebral PI3K-Akt pathway is reduced only by the loss of IRS-2.

3.4. IRS-2 deletion inhibited insulin-induced hippocampal Akt activation more strongly than IRS-1 deletion

To further address the effect of IRS-1/2 deletion on the IIS activity, the response of Akt phosphorylation to in vivo insulin stimulation was examined by direct hippocampal perfusion of insulin via reverse microdialysis. First, we examined whether the stimulation by this method showed a near-physiological effect on endogenous brain insulin signaling. Insulin levels increased significantly in the hippocampal
Fig. 2. Deletion of IRS-1 and IRS-2 increases tyrosine phosphorylation of the remaining IRS in the cerebral cortex of A7-Tg mice. A and B, levels of tyrosine-phosphorylated IRS-2 and IRS-1 in the cerebral cortex of 9-month-old IRS-1-deficient (A) and IRS-2-deficient (B) male A7-Tg mice, respectively. Cortical lysates were immunoprecipitated with antibodies against IRSs, followed by immunoblotting with anti-phosphotyrosine antibody. Relative levels of signal intensity are shown in the lower panels. C and D, levels of IRS proteins in the cerebral cortex of 9-month-old IRS-1-deficient (C) and IRS-2-deficient (D) male A7-Tg mice. Immunoblot (upper panels) and densitometric (lower panels) analyses are shown. α-Tubulin served as loading control. The immunoblots show the results of two representative animals for each genotype. Data are mean ± SEM (Irs1+/−;A7-Tg mice: n = 10, Irs1−/−;A7-Tg mice: n = 8, Irs2+/−;A7-Tg mice: n = 10, Irs2−/−;A7-Tg mice: n = 10). *, p < 0.05; ***, p < 0.001, unpaired t-test.
Deletion of IRS-2 but not IRS-1 reduces the basal activation of Akt in the brain. A, immunoblot analysis of Akt phosphorylated at Ser473, Thr308, total Akt and α-tubulin in cortical lysates of 9-month-old IRS-1-deficient male A7-Tg mice (Irs1−/−;A7-Tg mice: n = 10, Irs1+/−;A7-Tg mice: n = 8). B and C, levels of pAkt S473 (B) and pAkt T308 (C) were normalized to total Akt. D, levels of total Akt were normalized to α-tubulin. E, immunoblot analysis of pAkt S473, pAkt T308, total Akt and α-tubulin in cortical lysates of 9-month-old IRS-2-deficient male A7-Tg mice (Irs2−/−;A7-Tg mice: n = 10, Irs2+/−;A7-Tg mice: n = 10). F–H, relative levels of pAkt S473 (F), pAkt T308 (G), and total Akt (H) were analyzed as in (B–D). The immunoblots show the results of two representative animals for each genotype. Data are mean ± SEM. *, p < 0.05, unpaired t-test.

3.5. Deletion of IRS-1 did not alter the phosphorylation of soluble tau in the cerebral cortex in A7-Tg mice

Next, we aimed to examine the effects of IRS deletion on AD pathophysiology. Tau, a microtubule-binding protein, accumulates in neurons and causes neurodegeneration in the brain of a range of neurodegenerative diseases, including AD. These pathological changes are associated with hyperphosphorylation of tau at multiple serine and threonine residues, although the causal relationship between phosphorylation and abnormal aggregation is still controversial. Previous studies have suggested that deletion of IRS-2 increases tau phosphorylation in mouse brain, whereas the effect of IRS-1-deficiency has not been reported (Killick et al., 2009; Schubert et al., 2003). We therefore biochemically analyzed tau phosphorylation in the TBS-soluble fraction of cerebral cortex of 9-month-old IRS-1-deficient A7-Tg mice. IRS-1 deletion slightly increased the levels of bands detected by antibodies recognizing phosphorylation of Thr212, Ser214 and Thr231 in human tau (Fig. 5F–I). Previous studies using an independently established IRS-2-deficient mouse strain have shown that down-regulation of a phosphatase, PP2A, may be involved in the enhancement of tau phosphorylation (Killick et al., 2009; Schubert et al., 2003). However, in our study, the relative protein levels of PP2A were not affected by the genotype of IRS-2 in A7-Tg mice (Irs2+/−;A7, 1.00 ± 0.11; Irs2−/−;A7, 1.192 ± 0.14), suggesting that other mechanism(s) may be involved in...
IRS-1 deficiency did not affect the brain insulin signaling, our observations in brains of APP transgenic mice. Taken together with the findings that absence of IRS-1 had no significant effect on the Aβ accumulation in A7-Tg mice at 15 months of age. In sharp contrast to the previous observation in IRS-2 deleted A7-Tg mice, Aβ deposition at 15 months of age compared to control A7-Tg mice had significantly lower Aβ levels at 9 months of age and amyloid deposition at 15 months of age compared to control A7-Tg mice (Wakabayashi et al., 2019). We have chosen to evaluate the Aβ deposition in the brains of A7-Tg mice, the levels of both soluble and insoluble Aβ are gradually elevated with no visible amyloid plaques at 9 months of age, and amyloid plaques start to deposit after ~11 months of age. We previously demonstrated that IRS-2 deficiency in A7-Tg mice had significantly lower Aβ levels at 9 months of age and amyloid deposition at 15 months of age and amyloid deposition in the brains of IRS-1 deficient A7-Tg mice at these two time points. In the cerebral cortices of 9-month-old IRS-1 deficient mice after 1 h of insulin (400 nmol/l) or aCSF perfusion (irs1+/+, mice with aCSF: n = 5, irst1−/− mice with insulin: n = 6, irst1−/− mice with aCSF: n = 6, irst1−/− mice with insulin: n = 10). B and C, levels of pAkt S473 (B) and pAkt T308 (C) were normalized to total Akt. D, immunoblot analysis of pAkt S473, pAkt T308, and total Akt in hippocampal lysates of 4-month-old IRS-2-deficient A7-Tg mice after 1 h of insulin or aCSF perfusion (irs2+/+, A7-Tg mice with aCSF: n = 4, irst2−/−;A7-Tg mice with insulin: n = 5, irst2−/−;A7-Tg mice with aCSF: n = 4, irst2−/−;A7-Tg mice with insulin: n = 5). E and F, levels of pAkt S473 (E) and pAkt T308 (F) were normalized to total Akt. Data are mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001, one-way ANOVA with Tukey’s post-hoc test.

The increased phosphorylation of tau caused by IRS-2 deficiency.

3.6. Deletion of IRS-1 did not alter Aβ levels and the deposition of amyloid plaques in A7-Tg mice

We then investigated the effects of IRS-1 deletion on amyloid pathology. In the brains of A7-Tg mice, the levels of both soluble and insoluble Aβ are gradually elevated with no visible amyloid plaques at 9 months of age, and amyloid plaques start to deposit after ~11 months of age. We previously demonstrated that IRS-2 deficiency in A7-Tg mice had significantly lower Aβ levels at 9 months of age and amyloid deposition at 15 months of age compared to control A7-Tg mice (Wakabayashi et al., 2019). We have chosen to evaluate the Aβ pathobiology in IRS-1 deficient A7-Tg mice at these two time points. In the cerebral cortices of 9-month-old IRS-1 deficient mice after 1 h of insulin (400 nmol/l) or aCSF perfusion (irs1+/+, mice with aCSF: n = 5, irst1−/− mice with insulin: n = 6, irst1−/− mice with aCSF: n = 6, irst1−/− mice with insulin: n = 10). B and C, levels of pAkt S473 (B) and pAkt T308 (C) were normalized to total Akt. D, immunoblot analysis of pAkt S473, pAkt T308, and total Akt in hippocampal lysates of 4-month-old IRS-2-deficient A7-Tg mice after 1 h of insulin or aCSF perfusion (irs2+/+, A7-Tg mice with aCSF: n = 4, irst2−/−;A7-Tg mice with insulin: n = 5, irst2−/−;A7-Tg mice with aCSF: n = 4, irst2−/−;A7-Tg mice with insulin: n = 5). E and F, levels of pAkt S473 (E) and pAkt T308 (F) were normalized to total Akt. Data are mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001, one-way ANOVA with Tukey’s post-hoc test.

4. Discussion

IRS-1 and IRS-2 are key components of IIS, sharing a homologous molecular structure, similar tyrosine-phosphorylation motifs, and ubiquitous expression throughout the body. However, studies in knockout mice have revealed that the roles of IRS-1 and IRS-2 proteins in IIS are complementary, rather than redundant, in various organs (Kubota et al., 2017; White, 2002). With regard to their roles in the brain, tissue growth during development is likely to be more dependent on signaling through IRS-2 than IRS-1: IRS-2-deficient mice exhibited a relative decrease in brain size compared to body size, whereas analysis using IGF-1 overexpression in IRS-1-deficient mice shows little contribution of IRS-1 to IGF-1-dependent brain growth (Pete et al., 1999; Schubert et al., 2003; Ye et al., 2002). In relation to disease states in human brains, ex vivo stimulation analysis of human autopsy brains has shown that insulin receptor activation triggers IRS-1 and IGF-1 receptor activation triggers IRS-2 mediated signaling, respectively, and that resistance may occur in both pathways in the brain tissues from patients with AD (Talbot et al., 2012). However, previous knowledge on the relative in vivo contributions of IRS-1 and IRS-2 in the mature brain has been limited.

The role of IRS-2 in the adult brain has been shown in several genetically modified models. Mice conditionally deficient in IRS-2 in the hypothalamus and pancreatic β-cells exhibited obesity and leptin resistance (Choudhury et al., 2005; Kubota et al., 2004). Brain-specific IRS-2 deletion induced peripheral insulin resistance and prolonged lifespan, as well as constitutive reduction in the phosphorylation of Akt and GSK-3β...
in hippocampus (Costello et al., 2012; Taguchi et al., 2007). In mice systemically lacking IRS-2, however, alterations in the brain IIS have not been definitively elucidated (Freude et al., 2009; Killick et al., 2009; Schubert et al., 2003). Furthermore, no studies have evaluated IRS-2-dependent brain insulin responsiveness in vivo. In the present study, we showed that IRS-2 deficiency significantly suppressed the activation of insulin-Akt pathway in the cerebral cortices of A7-Tg mice. We simultaneously observed a compensatory increase in tyrosine-phosphorylated IRS-1, which did not fully restore the brain IIS activity, resulting in a decreased phosphorylation of Akt. Thus, IRS-2 deficiency is considered to lead to an insulin-resistant state in the cerebral cortices, where amyloid pathology is most prominent in human AD and model mice.

The effects of IRS-1 deficiency in the adult brain are less well understood compared to those of IRS-2, although a partial contribution to memory function has been suggested by knockdown experiments in rats (Sánchez-Sarásúa et al., 2021). By direct administration of insulin into the hippocampus by reverse microdialysis, we found a limited effect of IRS-1 deficiency on insulin-dependent Akt phosphorylation, especially on the Thr308 phosphorylation of Akt (Fig. 4C); moreover, in the cerebral cortex, the basal activation of Akt also was paradoxically increased. One possible explanation for this would be that IRS-2 was activated in a compensatory manner, which robustly restored the effects of IRS-1 deficiency on brain IIS. The reason for the particularly preserved phosphorylation of Thr308 is unknown. However, in the liver or skeletal muscles of mice doubly lacking IRS-1 and IRS-2, Thr308 phosphorylation by PDK1 was almost abolished, whereas phosphorylation of Ser473 by mTORC2 was partially preserved (Dong et al., 2008; Long et al., 2011). This may imply that phosphorylation of Thr308 can be interpreted as a direct indicator of the IRS-PI3K-PDK signaling axis. Taken together, our results suggest that IRS-2 is the major mediator of the IIS pathway in adult mouse brains.

Our study revealed a mutually compensatory increase in the basal tyrosine phosphorylation of IRS-1 or IRS-2 in the adult mouse brain, without alteration of the protein levels. IRS-1 and IRS-2 exhibited similar cellular expression patterns in astrocytes and neurons, which might have enabled the compensatory activation. One possible mechanism for the different effects of IRS-1 and IRS-2 deficiencies on the
activation of brain PI3K-Akt signaling, despite the fact that they can result in compensatory activation of each other, would be the different expression levels of IRS-1 and IRS-2. Although the qRT-PCR and biochemical analyses used in this study do not enable direct comparison of the mRNA or protein levels for each cell type, evaluation of transcript copy numbers using qRT-PCR against cDNA-based standards has suggested that mRNA expression of IRS-2 is higher than that of IRS-1 in the brain (Kleinridders et al., 2014). We have shown that IRS-2 deficiency caused a greater enhancement of tyrosine phosphorylation of the remaining IRS-1 compared with that of IRS-2 in IRS-1 deficiency (2.2-fold vs. 1.2-fold, Fig. 2); the fact that the loss of IRS-2 induced a more pronounced increase in the phosphorylation of IRS-1 might indicate the

**Fig. 6.** Deletion of IRS-1 does not alter Aβ levels and the deposition of amyloid plaques in A7-Tg mice. A and B, levels of Aβ40 and Aβ42 in TBS-soluble (A) and SDS-insoluble (B) fractions of the cerebral cortex of 9-month-old IRS-1-deficient male A7-Tg mice were analyzed by ELISA (Irs1<sup>+/+</sup>;A7-Tg mice: n = 10, Irs1<sup>−/−</sup>;A7-Tg mice: n = 8). C–E, immunohistochemical analysis of 15-month-old IRS-1-deficient female A7-Tg mouse brains using an anti-Aβ (82E1) antibody (Irs1<sup>+/+</sup>;A7-Tg mice: n = 10; Irs1<sup>−/−</sup>;A7-Tg mice: n = 9). Representative images of the brain regions including the parietal cortex and cingulate gyrus (C), hippocampus (D), and piriform cortex (E) are shown. Amyloid deposition was evaluated by quantitating the percentage of area covered by Aβ immunoreactivity (right panel of each figure). Data are mean ± SEM. Unpaired t-test.
preponderance of IRS-2 expression in the brain. Alternatively, it is also possible that the predominantly expressed IRS is different in each region of the brain. For example, in the liver, differential expression patterns of IRS-1 and IRS-2 within the organ have been reported to cause functional differences in insulin signaling (Kubota et al., 2016). Although the anti-amyloid effect of IRS-2 deficiency is widely recognized regardless of brain region, more detailed analysis of its regional expression profile may be necessary. In addition, differences in the relative activity of tyrosine-phosphorylated IRS-1 and IRS-2 at the basal level, or differences in systemic phenotypes upon IRS deletion may also influence the brain IIS.

Previous studies on APP transgenic mice lacking IIS-related genes, i.e., IRS-2, IR and IGF-1R, exhibited marked reduction in amyloid deposition, suggesting that reduced IRS may play a protective role against amyloid accumulation (Freude et al., 2009; Gontier et al., 2015; Killick et al., 2009; Stöhr et al., 2013; Wakabayashi et al., 2019). In sharp contrast, we showed that IRS-1 deficiency does not affect the AD-related changes including amyloid deposition and tau phosphorylation in Aβ-Tg mice. Our observation that deficiency of IRS-1 had a limited effect on brain IIS activity and did not affect the amyloid pathology, whereas deficiency of IRS-2 reduced brain IIS and strongly inhibited amyloid accumulation, may support the notion that the extent of amyloid deposition is proportional to the magnitude of the total brain IIS activity. Conversely, it is still possible that IRS-1 and IRS-2 have different downstream targets, and that the specific loss of IRS-2 and downstream target attenuates the amyloid pathology. There are, however, few findings supporting the physiological differences in the function of IRS-1 and IRS-2. Indeed, proteomic analysis of the phosphotyrosine interactors showed a large overlap of binding molecules between IRS-1 and IRS-2, suggesting a functionally redundant molecular property (Hanke and Mann, 2009). It is also possible that changes in the peripheral metabolic state caused by the loss of each IRS may have different effects on brain pathology. The anti-Aβ effect is likely to depend on changes in brain IIS, as it is also observed by neuron-specific IGF-1R deficiency without a clear peripheral phenotype, although the peripheral effect on tau pathology is still unknown (Gontier et al., 2015).

Peripheral and central IIS impairments have been implicated in anti-aging and longevity effects (Blüher et al., 2003; Cohen et al., 2009; Holzenberger et al., 2003; Selman et al., 2008; Taguchi et al., 2007): deletion of brain IGF-1 or systemic IRS-2 has been shown to reduce Aβ accumulation and protect against premature death, whereas reduction of Aβ in neuronal IR deletion was not associated with alteration in survival (Freude et al., 2009; Stöhr et al., 2013). Furthermore, activation or inhibition of neuronal FoxO1, a regulator of anti-aging signaling downstream of the insulin pathway, had no effect on the brain Aβ levels (Stöhr et al., 2013). Taken together with our present observation that deletion of IRS-1 did not alter amyloid deposition despite the documented extension of lifespan (Selman et al., 2008), it may be reasonable to speculate that the anti-amyloid effect of reduced IIS is independent of changes in longevity-related signals. The molecular mechanism whereby reduced IIS inhibits Aβ accumulation is an issue for future studies.

In conclusion, our observations suggest that IRS-2 plays a major role in brain IIS, which might have determined the difference in the effects of deletion of IRS-1 or IRS-2 on the brain amyloid pathology. Further analysis of the mediators downstream of IRS-1 or IRS-2 in brain IIS signaling that are responsible for the anti-amyloid effects upon deletion may enable us to better understand the pathophysiology of amyloid deposition in brains and develop therapeutic methodologies against AD pathology.

Author contributions

T.O., T.S., T.W., and T.I. designed the research; T.O., T.S., and T.N. performed experiments and analyzed data; N.K. provided the resources; T.O., T.W., and T.I. wrote the manuscript; N.K., T.K., T.W., and T.I. supervised the study.

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Declaration of Competing Interest

Toshitaka Ochiai is an employee of Kaken Pharmaceutical Co., LTD.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2021.105510.

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