Insulin deficiency promotes formation of toxic amyloid-β42 conformer co-aggregating with hyper-phosphorylated tau oligomer in an Alzheimer's disease model

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A B S T R A C T

The toxic conformer of amyloid β-protein (Aβ) ending at 42 (Aβ42), which contains a unique turn conformation at amino acid residue positions 22 and 23 and tends to form oligomers that are neurotoxic, was reported to play a critical role in the pathomechanisms of Alzheimer's disease (AD), in which diabetes mellitus (DM)-like mechanisms are also suggested to be operative. It remains to be established whether the attenuation of insulin signaling is involved in an increase of toxic Aβ42 conformer levels. The present study investigated the association between impaired insulin metabolism and formation of toxic Aβ42 conformers in the brains of an AD mouse model. In particular, we studied whether insulin deficiency or resistance affected the formation of toxic Aβ42 conformers in vivo. We induced insulin deficiency and resistance in 3xTg-AD mice, a mouse AD model harboring two familial AD-mutant APP (KM670/671NL) and PS1 (M146 V) genes and a mutant tau (P301L) gene, by streptozotocin (STZ) injection and a high fructose diet (HFuD), respectively. Cognitive impairment was significantly worsened by STZ injection but not by HFuD. Dot blot analysis revealed significant increases in total Aβ42 levels and the ratio of toxic Aβ42 conformer/total Aβ42 in STZ-treated mice compared with control and HFuD-fed mice. Immunostaining showed the accumulation of toxic Aβ42 conformers and hyper-phosphorylated tau protein (p-tau), which was more prominent in the cortical and hippocampal neurons of STZ-treated mice compared with HFuD-fed and control mice. HFuD-fed mice showed only a mild-to-moderate increase of these proteins compared with controls. Toxic Aβ42 conformers were co-localized with p-tau oligomers (Pearson’s correlation coefficient = 0.62) in the hippocampus, indicating their co-aggregation. Toxic Aβ42 conformer levels were inversely correlated with pancreatic insulin secretion capacity as shown by fasting immunoreactive insulin levels in STZ-treated mice and to a lesser extent in HFuD-fed mice compared with controls. These findings suggest that insulin deficiency rather than insulin resistance and the resultant impairment of brain insulin signaling facilitates...
1. Introduction

Alzheimer’s disease (AD) is a major cause of devastating dementia in older people. Two well-known pathological hallmarks are senile plaques and neurofibrillary tangles, of which amyloid β-protein (Aβ) and hyper-phosphorylated tau protein (p-tau) are the main constituents, respectively. Aβ40 is the major species of Aβ physiologically secreted in the brain while a minor species, Aβ42, is more aggregative and readily forms toxic oligomers (Ono, 2018).

In AD, mitochondrial dysfunction (Weidling and Swerdlow, 2019), inflammation (Dionisio-Santos et al., 2019), cerebral hypoperfusion (Di Marco et al., 2015) and insulin resistance (Rorbach-Dolata and Piwowar, 2019), are thought to play crucial roles via convoluted associations with each other. Specifically, metabolic alterations such as diabetes mellitus (DM) impair the insulin signaling pathway, suppressing phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-Akt, which may affect neurons, microglia, and astrocytes, leading to cognitive dysfunction in AD brains (Gabbouj et al., 2019). Thus, much attention has been paid to the findings that AD and DM share many pathological characteristics including impaired insulin sensitivity, Aβ accumulation, tau hyper-phosphorylation, brain vasculopathy, inflammation, and oxidative stress (Tumminia et al., 2018).

In AD brains, decreased insulin receptor mRNA and protein as well as reduced insulin receptor binding were found (Arnold et al., 2018). In particular, inhibitory phosphorylation of insulin receptor substrate 1, a biomarker for insulin resistance, was increased in the hippocampus and associated with Aβ plaque burdens and memory impairment (Talbot et al., 2012). In addition, intranasal insulin administration partially improved cognitive function in AD patients (Sabramanian and John, 2012; Claxton et al., 2013; Claxton et al., 2015; Arnold et al., 2018) and alleviated cognitive deficits in amyloid precursor protein (APP)/presenilin 1 (PS1) transgenic mice (Mao et al., 2016). Similarly, in 3xTg-AD mice, a mouse AD model harboring two familial AD-mutant APP (KM670/671NL and PS1 (M146 V) genes and a mutant Tau (P301L) gene, age-dependent glucose intolerance and pancreatic amyloidopathy were observed at 14 months of age (Vandal et al., 2015). In this model, a high fat diet (HFD) exaggerated glucose intolerance, brain soluble Aβ, and memory impairment, all of which were reversed by insulin injection (Vandal et al., 2014). Furthermore, it was also demonstrated that diet-induced insulin resistance aggravated cognitive dysfunction and Aβ deposition in Tg2576 mice (Ho et al., 2004) and that cross-breeding of APP23 (AD model) and ob/ob (DM model) mice exacerbated cognitive dysfunction, AD pathology and cerebrovascular inflammation (Takeda et al., 2010). These findings collectively suggest that impaired insulin signaling in neurons may worsen cognitive disturbance and pathologies in AD patients and mouse models. Thus, many anti-diabetic drugs have been investigated for AD, revealing some beneficial effects on cognitive function and AD pathologies (Rizvi et al., 2015). However, other reports indicated no relationship between DM and AD (Gratuze et al., 2016; Murakami et al., 2011). Interestingly, Clarke et al. (2015) recently reported that the intra-cerebroventricular infusion of Aβ oligomers affected hypothalamic neurons that induce peripheral glucose intolerance, suggesting Aβ oligomers may target brain regions involved in peripheral metabolic control. Furthermore, the loss-of-function of tau protein may induce brain insulin resistance (Marciniak et al., 2017). Thus, the association between AD and DM in brain and peripheral tissues remains complicated.

DM can be characterized as type 1 DM (T1DM) caused by insulin deficiency and type 2 DM (T2DM) associated with insulin resistance. Epidemiological surveys have repeatedly shown that T2DM increases AD risk nearly 2-fold (Ohara et al., 2011); however, an association between T1DM and AD remains unclear. Genes involved in T2DM were significantly altered in AD brains as well as 3xTg-AD mouse brains (Hokama et al., 2014). However, a relationship between T2DM and AD pathology in autopsied brain samples is almost uniformly negative (Arnold et al., 2018). In support of an association between T1DM and AD, levels of p-tau and Aβ42 in cerebrospinal fluid (CSF) were higher in T1DM than in controls while elevated CSF tau levels were also associated with decreased white matter integrity in T1DM patients (Ouwens et al., 2014). T1DM in monkeys induced by streptozotocin (STZ) injection increased the inhibitory phosphorylation of insulin receptor substrate 1 (IRS1), soluble Aβ, and p-tau in the hippocampus (Morales-Corralliza et al., 2016). The induction of T1DM by STZ in 4-month-old APP transgenic (Tg) mice also decreased insulin receptor activity in the brain and increased Aβ, p-tau and glycogen synthase kinase-3β (GSK-3β) activity, which was partially prevented by insulin therapy (Jolivalt et al., 2010). Therefore, insulin deficiency as well as insulin resistance may result in the attenuation of insulin signaling in cells.

Recently, a novel minor species of Aβ42 with a turn at positions 22 and 23 and a major species with a turn at positions 25 and 26 in wild type Aβ42 aggregates were reported (Masuda et al., 2009). The novel Aβ42 with a turn at positions 22 and 23 was reported to be more aggregative and neurotoxic than the normal form of Aβ42 (Murakami et al., 2016; Izzo et al., 2012; Murakami et al., 2016). This species is termed “toxic Aβ42 conformer”, which may be a novel therapeutic target (Izzo et al., 2017). We demonstrated that toxic Aβ42 conformers accumulated in neurons at an early stage in 3xTg-AD mice (Soejima et al., 2013). Furthermore, the ratio of toxic Aβ42 conformer to total Aβ42 in the CSF was significantly increased in patients with AD and those with mild cognitive impairment compared with age-matched controls (Murakami et al., 2016). However, the mechanisms involved in how toxic Aβ42 conformers are generated and accumulated in the AD brain remain to be determined. Particularly, it is unknown whether the formation of toxic Aβ42 conformers is influenced by insulin deficiency and resistance. In the present study, we aimed to clarify the relationship between impaired insulin metabolism and the formation of toxic Aβ42 conformers in the brains of a mouse AD model. We report that insulin deficiency rather than resistance in our models facilitates memory impairment and promotes the formation of toxic Aβ42 conformers in vivo, which co-aggregate with p-tau oligomers in hippocampal neurons.

2. Material and methods

2.1. AD model mice

3xTg-AD mice on a congenic C57BL/6 background (Oddo et al., 2003) were used as an AD model throughout all experiments. Mice were maintained on a 12-h light/12-h dark schedule. We used an injection of STZ, a structural analogue of N-acetyl glucosamine, to induce insulin deficiency (Gvazava et al., 2018) and feeding with a high fructose diet (HFuD) was used to induce insulin resistance in 3xTg-AD mice (Catena et al., 2003; Thorburn et al., 1989). The animals were divided into the following four groups: 1) STZ control group; a control diet with citrate buffer injection, 2) STZ group; a control diet with STZ injection, 3) HFuD control group; a control diet with no injection, and 4) HFuD group; HFuD with no injection. Each group contained 6 male and 6 female mice.

To induce insulin deficiency, 6-month-old 3xTg-AD mice were treated with 50 mg/kg STZ (Wako, Osaka, Japan) for 5 consecutive days (n = 12). STZ was quickly dissolved in citrate buffer (pH 4.5) and
intraperitoneally injected. STZ is taken up by pancreatic β-cells via GLUT2 leading to selective β-cell death by DNA fragmentation (Ventura-Sobrilla et al., 2011). To induce insulin resistance, mice were given free access to HFD (Oriental Yeast, Tokyo, Japan) containing 60% carbohydrate (fructose), 5.0% fat, and 17.8% protein as well as 10% fructose water from 6 to 9 months of age (n = 12), as previously reported (Catena et al., 2003;Thorburn et al., 1989). Fasting plasma glucose (PG) and immunoreactive insulin (IRI) were measured in mice before treatment at 6 months of age and after treatment at 9 months of age. The experimental design is summarized in Supplementary Fig. S1A. All experiments were approved by the ethical committee of Kyushu University.

2.2. Morris water maze (MWM) analysis

Short-term memory function was evaluated by the MWM test. A circular tank (90-cm diameter) was filled with water at 24 ± 1 °C. To evaluate spatial working memory, mice were trained to escape onto a 10-cm-diameter circular and clear platform submerged 1.5 cm beneath the surface of the water that was invisible to mice while swimming. Mice were given four consecutive trials per day for 3 days. The platform location was selected randomly for each mouse but was kept constant throughout training. Mice were placed into the tank at one of four designated start points. If a mouse failed to find the platform within 60 s it was manually guided to the platform and remained there for 10 s. To evaluate memory retrieval, a probe test was conducted 24 h after the last acquisition trial. In this test, the platform was removed, and mice started on the opposite side of the pool were allowed to swim for 60 s. An overhead camera recorded the swimming paths and the track was analyzed using a DV-Track Video Tracking System (Muromachi Kikai, Tokyo, Japan) as previously reported (Itimo et al., 2011; Nakamura et al., 2017). STZ treatment and HFD feeding started on the day after the first probe test. The same training and probe tests were conducted 12 weeks after each treatment in each group. The parameters measured by the probe trial were initial latency to cross the platform location (s), number of platform location crosses (n), and time spent in the quadrant containing the platform location (%).

2.3. Preparation of brain tissue

Mouse brain tissues were separated into two hemispheres. One hemisphere was fixed in 4% paraformaldehyde (PFA) and embedded in paraffin for immunofluorescent staining. The other hemisphere was frozen in liquid nitrogen, homogenized in a 10 × volume (w/v) of 50 mM Tris-HCl buffer (pH 7.6) containing 150 mM NaCl (TBS), a mixture of protease inhibitors (Complete™ Mini, Roche Diagnostics, Tokyo, Japan), and a mixture of phosphatase inhibitors (Phos STOP, Roche Diagnostics) supplemented with 0.7 μg/mL pepstatin A (Peptide Institute, Osaka, Japan) and 1 mM phenylmethyl-sulfonyl fluoride (Tokyo Chemical Industry, Tokyo, Japan). After sonication, the homogenates were centrifuged at 186,000 × g for 30 min at 4 °C using an Optima TLX ultracentrifuge and a TLA100.4 rotor (Beckman, Palo Alto, CA) to obtain supernatant (soluble) and pellet (insoluble) fractions. Protein concentrations were measured using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

2.4. Dot blot and western blot analysis

A conformation-targeted monoclonal antibody against the toxic Aβ42 conformer (11A1) was previously described (Murakami et al., 2010). 11A1 was generated against the E22P region, which is an artificial sequence mimicking the toxic turn conformation of Aβ42. Recently, another antibody against the same E22P conformer (24B3), which is more specific for the toxic turn conformation of Aβ42 compared with 11A1, was reported (Izu et al., 2017; Murakami et al., 2016). Therefore, for the quantification of soluble toxic Aβ42 conformer and total Aβ42 levels, we performed dot blots using three anti-Aβ antibodies, 12F4, 11A1, and 24B3, because dot blots are more suitable for quantitative assay than western blotting, in which anti-Aβ42 antibodies react with multiple bands (Murakami et al., 2010).

Levels of soluble toxic Aβ42 conformer, total Aβ42, and p-tau in TBS extracts were measured by a dot blotting assay system (Bio-Dot® Microfiltration Apparatus, Bio-Rad Laboratories, Hercules, CA). Anti-E22P (11A1) and anti-E22P (24B3) antibodies (Immuno-Biological Laboratories (IBL), Gunma, Japan) against toxic Aβ42 conformers, anti-Aβ42 (12F4) antibody (BioLegend, San Diego, CA) against the Aβ42 end, anti-phosphorylated-tau (Thr231) antibody (AT180, Thermo Fisher Scientific, Waltham, MA), anti-phosphorylated-tau (Ser202, Thr205) antibody (AT8, Thermo Fisher Scientific) against p-tau, and anti-total tau antibody (Tau46, Santa Cruz Biotechnology, Dallas, TX) were used as primary antibodies (Supplementary Table S1). The procedure was performed according to the manufacturer’s guidelines. Briefly, 2.5 μg soluble protein diluted in 100 μL TBS was applied to a Zeta-Probe membrane in duplicate. After blotting, the membrane was detached from the apparatus and blocked with 5% skim milk in TBS + 0.1% Tween 20 (TBST) for 1 h at room temperature, followed by incubation with each primary antibody (24B3 [1:200]; 11A1 [1:100]; 12F4 [1:1000]; and p-tau at Thr-231 [1:1000]) in Can Get Signal solution 1 (Toyobo, Osaka, Japan) overnight at 4 °C. After washing in TBST, the membrane was incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (Pierce) in Can Get Signal solution 2 (Toyobo) for 1 h at room temperature. After washing in TBST, specific dots were detected using Supersignal West Dura Extended Duration Substrate (Pierce). Intensities of the dots were measured using ImageJ software (https://imagej.nih.gov/ij/; National Institutes of Health, Bethesda, MD).

For western blot analysis, 10 μg of each total protein in loading buffer (20% glycerol, 4% SDS, 0.1 M Tris-Cl [pH 6.8], 12% mercaptoethanol, 0.01% bromophenol blue) was electrophoresed in a 4%-15% precast polyacrylamide gel (Bio-Rad) and electrotransferred onto a PVDF membrane (Millipore, Bedford, MA). The membrane was blocked with Blocking One or Blocking One-P (Nacala-l Tesque, Inc., Kyoto, Japan) for 30 min, followed by incubation with the appropriate antibodies (24B3 [1:100]; 11A1 [1:100]; 12F4 [1:1000]; p-IRS-1 [1:1000], C-terminal APP [1:1000], pro-IL-1β and IL-1β [1:1000]) in Can Get Signal solution 1 (Toyobo) overnight at 4 °C and with anti-β-actin antibody (1:5000) in TBST for 30 min at room temperature. After washing in TBST, the membrane was incubated with the appropriate secondary antibodies in Can Get Signal solution 2 (Toyobo) or TBST for 1 h at room temperature. Specific bands were detected using the Supersignal West Dura Extended Duration Substrate (Pierce). The band intensities of APP, p-IRS-1, and pro-IL-1β were measured using ImageJ software (National Institutes of Health).

2.5. Blood sampling, measurement of glucose and insulin, insulin tolerance tests (ITTs), and immunohistochemical/histological staining

Mice were fixed on a retractor and blood was collected from the tail tip. A glucometer (ACCU-CHECK Aviva*, Roche Diagnostics) commonly used in clinical practice was used to measure glucose levels. Plasma insulin levels were measured by an enzyme-linked immunosorbent assay (ELISA) using the Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Yokohama, Japan) according to the manufacturer’s guidelines. For ITTs, mice were fasted 6 h before the tests, and were then administered an intraperitoneal injection of regular human insulin (Novo Nordisk, Tokyo, Japan) at 0.50 U/kg. Blood samples were obtained from the tail blood vessels at 0, 15, 30, 45, 60, and 90 min, followed by measurement of glucose levels as described above. The blood glucose area under the curve (AUCgμ) was calculated by the trapezoid model using decreases in plasma glucose (PG) levels at various time points according to the following formula. PG levels before insulin injection
were used as the basal level of glucose. AUCgpus represents insulin tolerance. AUCgpus = \[30 \times (0 \text{ min PG} + 30 \text{ min PG}) + 2 \] + \[30 \times (30 \text{ min PG} + 60 \text{ min PG}) + 2 \] + \[30 \times (60 \text{ min PG} + 90 \text{ min PG}) + 2 \] + \[30 \times (90 \text{ min PG} + 120 \text{ min PG}) + 2 \].

For immunohistochemical staining, 4-μm-thick pancreas sections were incubated with primary anti-mouse insulin C monoclonal antibody (Clone M-Ins 1 J-4 [1:2000], Takara Bio Inc., Shiga, Japan) overnight at 4 °C, treated with an amino acid polymer conjugated to anti-mouse IgG Fab' labeled with horseradish peroxidase (Histofine Simple Stain Mouse MAX-PO (M), Nichirei, Tokyo, Japan), which was then developed with Histofine Simple Stain DAB solution (Nichirei). For histological staining, sections were stained with Masson's trichrome (MT) stain using standard protocols.

2.6. Immunofluorescent staining and measurement of the percentage area of immuno-positive staining

After the perfusion of mice with 4% PFA in PBS, the other brain hemisphere was immersed in 4% PFA in PBS for 48 h at 4 °C and embedded in paraffin. Brain tissues were cut into 4-μm-thick sections. To enhance the antigen-antibody reaction, the sections were treated with HistoVT One® (Nacalai Tesque) for 20 min at 90 °C. After washing three times in PBS + 0.01% Triton-X, the sections were incubated in the blocking solution Block Ace® (DS Pharma Biomedical, Osaka, Japan) for 30 min at room temperature, followed by incubation with the primary antibodies diluted in 1:10 with blocking solution overnight at 4 °C. We used anti-E22P antibody (11A1 [1:50], IBL), anti-phosphorylated tau at Thr-231 antibody (AT180 [1:500], Thermo Fisher Scientific), anti-inducible nitric oxide synthase (iNOS, NOS2) antibody (C-11 [1:100], Santa Cruz Biotechnology), and anti-ibl1 antibody (1:500, Wako) as the primary antibodies. Secondary antibodies were Alexa Fluor 488-conjugated goat anti-mouse IgG (green) (1:500, Invitrogen, Carlsbad, CA) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (red) antibodies (1:500, Invitrogen, Carlsbad, CA). The Vector TrueVIEW Autofluorescence Quenching Kit® (Vector Laboratories, Burlingame, CA) was used to inhibit autofluorescence to reduce background staining. For nuclear staining, antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) was added, followed by mounting with coverslips. The immuno-staining was observed using a BX50 Fluorescence Microscope (Olympus, Tokyo, Japan).

To measure the percentage immuno-positive area of Iba1 and NOS2, random non-overlapping regions of the cerebral cortices and hippocampus in each brain section were selected and analyzed using ImageJ software (National Institutes of Health). Each image was converted to an 8-bit image and then processed using threshold filters to detect the stained areas only, and the percentage immuno-positive area was then measured.

2.7. Confocal laser scanning microscopy and co-localisation analysis

The paraffin blocks were sliced into 4-μm-thick sections, deparaffinized with xylene, and rehydrated with decreasing concentrations of ethanol in water. Antigen retrieval was achieved using HistoVT One® (Nacalai Tesque) for 20 min at 90 °C. After washing three times in PBS + 0.01% Triton-X, the sections were incubated in the blocking solution Block Ace® (DS Pharma Biomedical) for 30 min at room temperature, followed by incubation with the primary antibodies diluted in 1:10 with blocking solution overnight at 4 °C. The sections were stained with anti-E22P antibody (11A1 [1:50], IBL) and anti-tau oligomers (T22 [1:500], Merck Millipore, Darmstadt, Germany) as primary antibodies. Secondary antibodies were the Alexa Fluor 488-conjugated goat anti-mouse IgG (green) (1:500, Invitrogen) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (red) antibodies (1:500, Invitrogen) diluted 1:10 with blocking solution. The sections were incubated for 2 h at room temperature. The Vector TrueVIEW Autofluorescence Quenching Kit® (Vector Laboratories) was used to inhibit autofluorescence to reduce background staining. For nuclear staining, antifade mounting medium with DAPI (Vector Laboratories) was added, followed by mounting with coverslips. Images were captured using a confocal laser microscope system (Leica TCS SP8 confocal microscope; Leica, Germany). An intensity line profile was measured using LAS X software (Leica). Images were imported into the Fiji version (http://fiji.sc) of the image processing software ImageJ. Fiji contains several pre-installed plugins. Pearson's coefficient was calculated using the colo2 plugin. Non-transgenic C57BL/6 mouse brain of a similar age was used as a negative control for T22 immunostaining.

2.8. Sandwich enzyme-linked immunosorbent assay and correlation study

Levels of toxic Aβ42 conformers in brain tissues were measured by ELISA kits (IBL) according to the manufacturer's guidelines. Briefly, 5 μg of protein diluted in 100 μL EIA buffer was applied to microtiter plates (96 wells) coated with anti-human Aβ N-terminus (82E1) antibodies with two or more epitopes and incubated overnight at 4 °C. After washing four times with PBS-T, each well was treated with 100μL of horseradish peroxidase-conjugated 2483 for 1 h at 4 °C. ELISA signals were detected by chemiluminescence using a substrate, 3,3’,5,5’-tetramethylbenzidine (TMB), and then measured with a microplate reader (Corona Electric Co., Katsuta, Japan). Correlation between the fasting IRI and toxic Aβ42 conformer levels were investigated by Pearson's correlation analysis.

2.9. Time-resolved fluorescence resonance energy transfer (TR-FRET) assay

Levels of Akt, p-Akt, glycogen synthase kinase-3β (GSK-3β), and p-GSK-3β were measured by a homogeneous time-resolved fluorescence assay using an HTRF® kit (Cisbio, Chiba, Japan) according to the manufacturer's instructions (Degorce et al., 2009). Each 5-μg sample was diluted in 16 μL TBS buffer and loaded into 384-well plates, followed by the addition of 4 μL detection reagent mixture of a d2-dye-conjugated Akt (FRET acceptor) and anti-phospho AKT-Eu cryptate (FRET donor) in each well. After incubation for 4 h at room temperature, the assay plates were measured using a plate reader in HTRF® detection mode. The results are expressed as a ratio of the acceptor fluorescence intensity (665 nm) divided by the donor fluorescence intensity (615 nm). The same procedure was used to measure the levels of p-Akt, GSK-3β, and p-GSK-3β.

2.10. Statistical analysis

All data were analyzed by R software version 3.4.2 for Windows (https://www.r-project.org/), and are expressed as the mean ± SEM (group n = 12 per group). To examine statistically significant differences between treatments and between time points in the MWM test, two-way ANOVA was used. Because the two animal models (STZ-treated and HFuD-fed mice) were induced using different methods, the MWM behavior analysis by ANOVA was performed in two parts (STZ control vs STZ, and HFuD control vs HFuD). When significant interactions were detected, post hoc paired comparisons before and after treatment were performed with a one sample t-test. Differences in continuous training for acquisition in the MWM test were examined using a repeated measures ANOVA. In biochemistry and immunostaining analyses, differences between the two groups were analyzed using an unpaired two-tailed Student’s t-test, and multiple comparisons were analyzed using the Tukey–Kramer test. Values of p < .05 were deemed to be statistically significant.
3. Results

3.1. Insulin secretion capacity is decreased in STZ-treated 3xTg-AD mice and insulin resistance is increased in HFuD-fed 3xTg-AD mice

As shown in Fig. 1A, FPG was significantly increased in STZ-treated 3xTg-AD mice but not in HFuD-fed 3xTg-AD mice. In addition, the fasting insulin levels were significantly decreased in STZ-treated mice, indicating that STZ treatment successfully induced insulin deficiency (T1DM) (Fig. 1B). By contrast, both FPG and the fasting IRI were not significantly altered in HFuD-fed mice (Fig. 1A, B). However, ITTs demonstrated that insulin reactivity was well preserved in STZ-treated mice but was significantly impaired in HFuD-fed mice (Fig. 1C), which was confirmed by comparing the AUCglu before and after treatment (Supplementary Fig. S2). Pathology of mouse pancreas and liver revealed that the number of pancreatic β-cells and insulin immunoreactivity were decreased in STZ-treated mice compared with HFuD-fed and control mice (Fig. 1D). Fructose is readily absorbed and rapidly metabolized in the liver, which stimulates de novo lipogenesis; therefore, plasma triglyceride (TG) levels were increased by STZ treatment and HFuD feeding (Supplementary Fig. S3). Synthesized TG leads to hepatic insulin resistance and dyslipidemia (Basciano et al., 2005) and numerous fat droplets were observed in the livers of HFuD-fed mice by histology (Fig. 1D, right panel). This finding indicates increased insulin resistance in HFuD-fed mice, consistent with previous reports (Nagata et al., 2004; Sumiyoshi et al., 2006).

3.2. Memory impairment is exacerbated in STZ-treated 3xTg-AD mice but not HFuD-fed 3xTg-AD mice

3xTg-AD mice develop memory impairment from 4 months of age, which coincides with the accumulation of Aβ in neurons (Billings et al., 2005). We studied the memory function of mice at 6 and 9 months of age. Consistent with a previous report (Yang et al., 2018), the percentage of time spent in the target quadrant was significantly lower for 3xTg-AD mice (25%–30%) than for non-transgenic C57BL/6 mice (35%–40%) at 6 months of age (Supplementary Fig. S1B), indicating significant memory impairment in 3xTg-AD mice. First, in 3xTg-AD mice, we observed that acquisition was significantly worsened in STZ-treated mice (p < .05, Fig. 2A) but not in HFuD-fed mice. Next, we found a significant decrease in the percentage of time spent in the platform quadrant (p < .05, Fig. 2B; data comparing the time spent in the opposite side quadrant are shown in Fig. S1C) and a significant increase in latency to platform (p < .05, Fig. 2C) only in STZ-treated mice.
mice. Crossing counts were also decreased in STZ-treated mice, although this was not statistically significant (Fig. 2D). In addition, when male and female mice were separately analyzed, the significant decrease in the percentage of time spent in the target platform quadrant was similar between STZ-treated male and female mice (Supplementary Fig. S1D). Therefore, peripheral insulin deficiency promotes a more marked cognitive decline compared with peripheral insulin resistance in 3xTg-AD mice.

3.3. Aβ42 accumulation, formation of toxic Aβ42 conformers and accumulation of p-tau are enhanced in the brains of STZ-treated 3xTg-AD mice compared with HFuD-fed 3xTg-AD mice

We measured the levels of Aβ42, toxic Aβ42 conformers and p-tau using dot blot analysis of the soluble fraction. Fig. 3A shows a representative dot blot analysis. Measurement of the blot intensity revealed significant increases in total Aβ42 levels (12F4/β-actin) and the ratio of toxic Aβ42 conformer/total Aβ42 (24B3/12F4 and 11A1/12F4) in STZ-treated 3xTg-AD mice compared with control and HFuD-fed 3xTg-AD groups (Fig. 3B, upper and middle left panels). By contrast, the ratios of 11A1/12F4 and 24B3/12F4 in HFuD-fed 3xTg-AD mice were only slightly increased compared with control 3xTg-AD mice. Furthermore, the p-tau levels (AT180 and AT8) were significantly increased in STZ-treated 3xTg-AD mice and, to a lesser extent, in HFuD-fed 3xTg-AD mice (Fig. 3B, lower left panel). However, total tau level (Tau46) was not significantly altered (Fig. 3B, lower right panel). We studied western blot analysis using these anti-Aβ antibodies. All three antibodies recognized two bands at 12 kD and 16 kD, which may correspond to the 3-mer and 4-mer of Aβ42, respectively (Fig. 3C, arrows). Extra-bands over 20 kD might represent Aβ oligomers. Mild differences in the intensities of the putative 3-mer and 4-mer in each western blot may be consistent with the results of the dot blots. Expressions of APP and its C-terminal fragment were not altered, suggesting that increases in Aβ42 levels by STZ treatment were associated with the decreased degradation, but not generation, of Aβ42 (Supplementary Fig. S4).

In addition, immunofluorescent staining with 11A1 showed a greater accumulation of toxic Aβ42 conformers in the cortical and hippocampal neurons of STZ-treated 3xTg-AD mice compared with HFuD-fed and control 3xTg-AD mice (Fig. 4A). Immunofluorescent staining with AT180 showed the highest accumulation of p-tau in the cortical and hippocampal neurons of STZ-treated 3xTg-AD mice, and a slightly lower accumulation in HFuD-fed 3xTg-AD mice compared with control 3xTg-AD mice (Fig. 4C). Immunofluorescent staining with AT8 also showed similar patterns, although the staining was weaker than that of AT180 because of its higher sensitivity to phosphatase activity (Supplementary Fig. S5). Quantitative analyses revealed significantly increased immunostaining-positive areas (%) by 11A1 and AT180 in the cortices and hippocampus of STZ-treated and HFuD-fed 3xTg-AD mice compared with control (STZ) 3xTg-AD mice (Fig. 4B and D), suggesting the accumulation of toxic Aβ42 conformer and p-tau in STZ-treated 3xTg-AD mice and, to a lesser extent, HFuD-fed 3xTg-AD mice compared with control (STZ) 3xTg-AD mice. These data are consistent with the results of the dot blot analysis mentioned above.

We next performed immunofluorescent staining with 11A1 and anti-tau oligomer antibody (T22) (Lasagna-Reeves et al., 2012). Fig. 5 demonstrates the double immunostaining of these proteins in the hippocampal area of STZ-treated 3xTg-AD mice. Immunostaining of 11A1
merged with T22 (red) demonstrated their co-localization (yellow) while T22 did not stain STZ-treated non-transgenic C57BL/6 mouse brain tissues (Supplementary Fig. S6). Dot-like features and relative intensity of fluorescences shown by the red arrow indicate the co-aggregation of these two proteins in neurons (Fig. 5D, E). Pearson’s correlation coefficient ($R = 0.62$) indicated a reasonably strong correlation between toxic Aβ42 conformers and p-tau. Although such pathology would be unique for 3xTg-AD mice, which harbor human P301L mutant MAPT genes, formation of the toxic Aβ42 conformer may contribute to the formation of p-tau oligomers in hippocampal neurons.

(no significant correlation between toxic Aβ42 conformer levels and fasting IRI was observed in each control group (Fig. 6A, C), toxic Aβ42 conformer levels were inversely correlated with fasting IRI in STZ-treated 3xTg-AD mice (correlation coefficient $= -0.5879$, $p = 0.04441$) (Fig. 6B). These data suggest that a decrease in serum insulin levels correlates with toxic Aβ42 conformer formation. However, no significant correlation was found between them in the HFuD-fed 3xTg-AD mouse group (Fig. 6D). Thus, peripheral insulin resistance may not be strongly associated with toxic Aβ42 conformer formation.

3.5. Insulin signaling is impaired in the brain tissues of STZ-treated and HFuD-fed 3xTg-AD mice

Next, we studied whether insulin signaling was impaired by decreased peripheral insulin levels and increased insulin resistance. The stimulation of insulin receptors involves the phosphoinositide 3-kinase/ Akt pathway and ultimately GSK-3β. Akt is activated by the phosphorylation of threonine 308 (Thr308) and serine 473 (Ser473) in the C-terminal hydrophobic motif (Alessi et al., 1996). Stimulation of the
insulin receptor inactivates GSK-3β via the phosphorylation of serine 9 (Ser9) (Jope and Johnson, 2004). Therefore, we measured Ser473-

Fig. 4. Immunofluorescent staining of toxic Aβ42 conformers and p-tau in 3xTg-AD mouse brain. Immunofluorescent staining of toxic Aβ42 conformers (11A1, green, A) and p-tau (AT180, green, C) in cortices (upper panels) and hippocampus (lower panels) in 3xTg-AD mice, and immunostaining-positive areas (%) of 11A1 (B) and AT180 (D). Immunostaining-positive areas were significantly increased in STZ-treated and HFuD-fed 3xTg-AD mice compared with control (STZ) 3xTg-AD mice, suggesting the accumulation of toxic Aβ42 conformer and p-tau by STZ treatment and HFuD feeding. This accumulation was more prominent in STZ-treated 3xTg-AD mice than in HFuD-fed 3xTg-AD mice. *p < .05, **p < .01, ***p < .001, n = 12/group. DAPI was used to stain nuclei (blue). Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Double immunofluorescent staining of toxic Aβ42 conformers and tau oligomers in STZ-treated 3xTg-AD mouse brain. Double immunofluorescent staining of toxic Aβ42 conformers (11A1, green), and tau oligomers (T22, red) with DAPI (blue) in STZ-treated 3xTg-AD mice at 9 months of age. A-D: 11A1 and T22 staining was merged and is represented as dot-like structures (yellow) in the cytoplasm. Scale bars = 10 μm. E: Relative intensity of fluorescence along the red arrow in the magnified view of D. Distance in the x-axis indicates the distance from the starting point of the red arrow in the magnified view of D. Co-localization of toxic Aβ42 conformers and tau oligomers was present in relatively large structures that may indicate aggregation, suggesting toxic Aβ42 conformers may co-aggregate with tau oligomers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
phosphorylated Akt (p-Akt) and Ser9-phosphorylated GSK-3β (p-GSK-3β) in brain tissues using an HTRF® assay (Degorce et al., 2009). As shown in Fig. 7A and C, p-Akt and p-GSK-3β levels were both significantly lower in STZ-treated and HFuD-fed 3xTg-AD mice compared with each control mouse group. However, total Akt and total GSK-3β levels were not altered by STZ treatment or HFuD feeding (Fig. 7B, D). Furthermore, decreased levels of p-Akt and p-GSK-3β were significantly lower in STZ-treated 3xTg-AD mice than in HFuD-fed 3xTg-AD mice (Fig. 7A, C). These data indicate that the inhibition of insulin signaling in brain tissues is more prominent in mice with insulin deficiency (STZ treatment) than with insulin resistance (HFuD feeding), consistent with the data of memory impairment (Fig. 2) and the accumulation of Aβ and p-tau (Figs. 3–5). In addition, to confirm the impaired insulin signaling in these mice, we performed western blotting of Serine307-phosphorylated IRS1 (pS307-IRS1), a specific marker of insulin resistance (Sud et al., 2016). The pS307-IRS1 levels were significantly elevated in both STZ-treated (p < .05) and HFuD-fed (p < .01) 3xTg-AD mice compared with each control 3xTg-AD mouse group, and were higher in HFuD-fed mice compared with STZ-treated mice (p < .05) (Fig. 7E).

3.6. Microglia are activated in brain tissues of STZ-treated and HFuD-fed 3xTg-AD mice

Finally, we immunostained microglial cells in the brain tissues of 3xTg-AD mice. Immunostaining with anti-Iba1 antibody revealed an increase in the number of microglial cells in STZ-treated and HFuD-fed 3xTg-AD mice (Fig. 8A). The mean Iba1-positive area in the cortex and hippocampus was significantly increased in STZ-treated 3xTg-AD mice, and to a lesser extent in HFuD-fed 3xTg-AD mice, compared with control 3xTg-AD mice (Fig. 8B). Moreover, immunostaining with anti-NOS2 antibody showed an increase in the number of NOS2-positive cells in STZ-treated and HFuD-fed 3xTg-AD mice compared with control 3xTg-AD mice (Fig. 8C). The mean NOS2-positive area was significantly greater in STZ-treated and HFuD-fed 3xTg-AD mice compared with control 3xTg-AD mice (Fig. 8D). In addition, we measured pro-IL-1β in mouse brains by western blot because mature IL-1β, which is rapidly secreted from cells following the caspase-1-dependent processing of pro-IL-1β ((Brough and Rothwell, 2007), has a very short half-life (Kudo et al., 1990). We found that pro-IL-1β levels were significantly increased in STZ-treated, but not HFuD-fed, 3xTg-AD mouse brains, compared with control mouse brains (Supplementary Fig. S7). Double immunostaining of Iba1 and the purinergic receptor P2Y12 (P2RY12), a specific marker for central nervous system (CNS) resident microglia (Mildner et al., 2017), revealed the frequent co-localization of Iba1 and P2RY12, suggesting that these Iba1-positive cells were CNS resident microglia (Supplementary Fig. S8). Because microglia were reported to be activated by Aβ (Dhawan et al., 2012; Bhaskar et al., 2014), impaired insulin signaling and the accumulation of toxic Aβ42 conformers may accelerate AD-like microglial activation.
4. Discussion

In the present study, we successfully induced insulin deficiency by STZ injection and insulin resistance by HFuD feeding; we found that insulin deficiency by STZ caused memory impairment and facilitated the formation of toxic Aβ42 conformers and p-tau oligomers concurrent with the activation of microglia. Our results are consistent with STZ-T1DM in monkeys (Morales-Corraliza et al., 2016) and in 4-month-old APPV171 + swe transgenic mice (Jolivalt et al., 2010). However, our results partly conflict with a previous study reporting that STZ-T1DM in 12-month-old 3xTg-AD mice increased amyloid plaques but did not affect memory deficit or p-tau accumulation (Hayashi-Park et al., 2017). This difference might be explained by the timing of STZ treatment: we applied STZ at 6 months of age. The induction of STZ-T1DM at a younger age may have more pronounced effects on the clinical manifestations and pathologies of AD, which is also supported by the worsening of memory impairment and p-tau accumulation by STZ-T1DM in 4-month-old APP transgenic mice (Jolivalt et al., 2010).

In this study, we used two antibodies, 11A1 and 24B3, against the toxic Aβ42 conformer. 24B3 is the more specific of the two antibodies because 11A1 recognizes wild type Aβ42 in addition to E22P-Aβ42 with toxic turn conformation (Murakami et al., 2010). Indeed, 24B3 was reported to be more specific for the toxic turn conformation of Aβ42 than 11A1 (Murakami et al., 2016), and it is suitable for ELISAs. The results of dot blots and western blots using 11A1 were similar to those using 24B3 (Fig. 3). These findings suggest that the majority of accumulated Aβ42 is the toxic conformer, although future studies should discriminatively assay for the toxic and non-toxic Aβ42 conformers.

Importantly, the formations of toxic Aβ42 conformers and p-tau were more pronounced in STZ-treated mice than in HFuD-fed mice. Because the perturbation of insulin signaling and impaired glucose metabolism were more pronounced in STZ-treated mice than in HFuD-fed mice, the difference between STZ and HFuD treatments may be partly explained by the difference in inhibitory effects on insulin signaling between the two treatments. However, the amount of toxic Aβ42 conformer had a significant negative correlation with pancreatic insulin secretion capacity as shown by fasting IRI levels in STZ-treated 3xTg-AD mice but not HFuD-fed 3xTg-AD mice in our study. This suggested that insulin deficiency induced by STZ treatment was more critical for toxic Aβ conformer formation than insulin resistance induced by HFuD treatment, although severely impaired glucose metabolism in STZ-treated mice might also be contributory.

Although the mechanism of toxic Aβ42 conformer formation remains to be established, we present a hypothesis in Supplementary Fig. S9. The greater attenuation of neuronal insulin signaling in STZ-treated mice compared with HFuD-fed mice downregulated insulin-degrading enzyme (Zhao et al., 2004), a major Aβ-degrading enzyme (Kurochkin et al., 2018), leading to Aβ42 accumulation. Increased Aβ42 may contribute to the formation of toxic Aβ42 conformers in STZ-treated mice. Toxic Aβ42 conformers were previously detected in neurons rather than the extracellular space in AD brains (Murakami et al., 2010) as well as in the neurons of 3xTg-AD mice even at an early stage (Soejima et al., 2013). Thus, decreased neuronal insulin signaling is likely to induce neuronal toxic Aβ conformers through decreased insulin-degrading enzyme. It was recently reported that the assembly of Aβ42 rather than Aβ40 was more sensitive to chiral substitutions, which affected monomer folding and assembly (Hayden et al., 2017). Furthermore, heterotypic interactions of Aβ peptides were shown to promote the generation of amyloid fibrils (Do et al., 2018). Collectively, these findings suggest that Aβ42 folding leading to the formation of
Aβ42 conformers is critical in amyloid fibril assembly, and that intermolecular interactions with various kinds of Aβ conformers may accelerate amyloidogenesis.

In addition, reduced insulin signaling upregulates dephosphorylated GSK-3β, which promotes p-tau formation (Supplementary Fig. S9). In corroboration, Ser9 phosphorylation of GSK-3β is regulated by PI3K/Akt such as in the insulin signaling pathway (Llorens-Martín et al., 2014), and impairment of the PI3K/Akt/GSK-3β pathway activates GSK-3β, resulting in the promotion of p-tau formation (Zhang et al., 2018). Therefore, it is also possible that tau pathology is in part promoted by reduction of insulin signaling independent of Aβ. Furthermore, the co-localization of toxic Aβ42 conformers and p-tau oligomers in relatively large structures, as seen in our study, might represent the co-aggregation of these proteins. Although the precise mechanism of interaction between Aβ and p-tau is unclear, three hypotheses are proposed: i) direct interaction within neurons, ii) indirect mechanisms via the induction of inflammation by glial cells, and iii) cross-seeding between misfolded protein species (Stancu et al., 2014). The third theory suggests that Aβ may act as a seed that transforms "mild tau strains" to "tau aggregates". Recent studies demonstrated that following the injection of human AD brain-derived pathological tau into mutated APP NFL knock-in mouse brains that do not overexpress tau, Aβ plaques facilitated neuritic tau aggregates (He et al., 2018), and that the inoculation of pancreatic islet amyloid polypeptide (IAPP) induced the cross-seeding of Aβ in vitro and in vivo, and exacerbated memory impairment in APP/PS1 mice (Thériault et al., 2016) and APP/Swe mice (Vandal et al., 2014; Thériault et al., 2016; Sah et al., 2017; Walker et al., 2017). The effects of HFD vary according to which mouse AD model is used and the timing of the introduction of HFD. In the present study, we used HFD instead of HFD to induce peripheral insulin resistance in 3xTg-AD mice. HFuD-fed mice showed increased peripheral insulin resistance; however, the elevation of FPG levels was minimal and did not reach the overt diabetic range. Nonetheless, HFuD induced mild insulin resistance in the brain and mild but significant increases in toxic Aβ conformers. In the case of HFuD, total Aβ42 did not increase while p-tau was markedly increased. This suggested that HFuD might facilitate toxic Aβ conformer production via the cross-seeding by p-tau oligomers. However, HFuD did not induce a significant exacerbation of cognitive dysfunction and AD-associated pathology compared with STZ treatment. One possible explanation is that fructose also contributes to the energy supply that activates neurons in specific brain regions such as the
hippocampus (Oppelt et al., 2017). In addition, fructose-1,6-bisphosphate, an intermediate product of fructose metabolism, inhibits NOS2 expression (Cárdenas et al., 2000), which may be beneficial against Aβ deposition and AD disease progression (Nathan et al., 2005). Thus, the deleterious effects of HFuD feeding on p-tau production may be counteracted by the above-mentioned beneficial effects of fructose.

Brain glucose transporter (GLUT) levels are also altered in AD brains. GLUT1 and 3 levels were decreased whereas GLUT2 levels were increased in AD brains (Liu et al., 2008). GLUT1 and 3 are expressed in neurons whereas GLUT2 is expressed in astrocytes (Szablewski, 2017). Interestingly, the intracerebroventricular injection of STZ induced astrocytic activation and co-expression of GLUT2 and insulin receptor (Knezovic et al., 2017). Therefore, peripheral STZ treatment might enhance neuroinflammation similar to that in AD brains. Thus, GLUT2 expression is worth investigating in our 3xTg-AD mice treated with STZ or HFuD in the future.

There were several limitations in this study. First, 3xTg-AD mice harbor the mutant human TAU gene; therefore, tau protein may aggregate more readily than in non-Tg mice and sporadic AD patients. To confirm the validity of the present findings, we are further investigating other types of AD model mice and brain tissues from AD patients. Second, STZ-treated and HFuD-fed mice may not be exact models of human T1DM and T2DM, respectively. In particular, HFuD feeding may induce various metabolic changes other than insulin resistance (Ormas et al., 2015). Nevertheless, because we confirmed the occurrence of insulin deficiency and resistance in STZ-treated and HFuD-fed mice, respectively, we think that the differential effects of insulin deficiency and resistance on toxin Aβ conformer formation reported in the present study will aid future studies investigating the relationship between AD and T1/T2DM. Third, although we demonstrated a correlation between formation of toxic Aβ42 conformers and insulin signaling, various pathogenic mechanisms, such as insulin signaling, inflammation, and oxidative stress, may be related to each other. The effects of these complicated mechanisms on toxic Aβ42 conformers should be carefully investigated in future studies. Fourth, we did not examine gender effects on glucose metabolism. However, because we used the same number of male and female mice in each group throughout the present experiments, we believe that gender effects on group comparisons should not severely distort the present results. Fifth, in the present study, we analyzed toxic Aβ42 conformers and p-tau in soluble fractions, but not in insoluble fractions. 11A1 and 24B3 antibodies recognize 3-mer or 4-mer of Aβ42, which are soluble. Because 11A1 and 24B3 antibodies recognize the specific turn conformation but not the primary structure of the peptide, toxic turn Aβ conformer may not be accurately measured by these turn conformation-specific antibodies in the insoluble fraction. Instead, we performed a quantitative immunofluorescent analysis of toxic Aβ42 conformers (11A1) and p-tau, which revealed a substantial increase in toxic Aβ42 conformers and p-tau, suggesting that co-aggregating structures of these proteins may exist as insoluble aggregates. We consider that analyzing specific Aβ conformer and p-tau in insoluble fractions is necessary in the future.

Finally, according to the present study, impaired insulin signaling in AD neurons might be a promising therapeutic target to improve dementia. The stimulation of insulin signaling in neurons might inhibit the formation and co-aggregation of toxic Aβ42 conformers and p-tau oligomers. We previously reported that apomorphine treatment improved memory function and AD pathology in 3xTg-AD mice (Himeno et al., 2011) and neuronal insulin resistance in the 3xTg-AD mouse brain (Nakamura et al., 2017). Therefore, therapies improving impaired insulin signaling in AD are worth investigating in future clinical trials.
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