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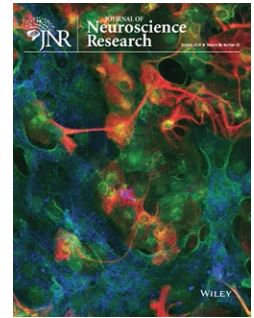Anderson, Nicholas J
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RESEARCH ARTICLE

Insulin deficiency, but not resistance, exaggerates cognitive deficits in transgenic mice expressing human amyloid and tau proteins. Reversal by Exendin-4 treatment

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Abstract

Epidemiological studies have pointed at diabetes as a risk factor for Alzheimer's disease (AD) and this has been supported by several studies in animal models of both type 1 and type 2 diabetes. However, side-by-side comparison of the two types of diabetes is limited. We investigated the role of insulin deficiency and insulin resistance in the development of memory impairments and the effect of Exendin-4 (Ex4) treatment in a mouse model of AD. Three–4-month-old female wild type (WT) mice and mice overexpressing human tau and amyloid precursor protein (TAPP) were injected with streptozotocin (STZ) or fed a high-fat diet (HFD). A second study was performed in TAPP-STZ mice treated with Ex4, a long-lasting analog of GLP-1. Plasma and brain were collected at study termination for ELISA, Western blot, and immunohistochemistry analysis. Learning and memory deficits were impaired in TAPP transgenic mice compared with WT mice at the end of the study. Deficits were exaggerated by insulin deficiency in TAPP mice but 12 weeks of insulin resistance did not affect memory performances in either WT or TAPP mice. Levels of phosphorylated tau were increased in the brain of WT-STZ and TAPP-STZ mice but not in the brain of WT or TAPP mice on HFD. In the TAPP-STZ mice, treatment with Ex4 initiated after established cognitive deficits ameliorated learning, but not memory, impairments. This was accompanied by the reduction of amyloid β and phosphorylated tau expression. Theses studies support the role of Ex4 in AD, independently from its actions on diabetes.

KEYWORDS
adiponectin, Alzheimer's disease, diabetes, GLP-1 analog, RRID:AB_94944, RRID:AB_476730, RRID:AB_570891, RRID:AB_662807

1 | INTRODUCTION

Diabetes is now clearly defined as a risk factor for Alzheimer's disease (AD) with epidemiological studies demonstrating a link between diabetes and AD (Arvanitakis, Wilson, Bienias, Evans, & Bennett, 2004; Craft, 2007; Luchsinger & Gustafson, 2009; Toro, Schonknecht, & Schroder, 2009). Studies describe both encephalopathy (loss of neurons) associated with cognitive deficits and myelopathy (myelin damage) in the central nervous system of patients suffering from type 1 or type 2 diabetes (Cukierman, Gerstein, & Williamson, 2005; Manschot et al., 2006; Ott et al., 1996; Reaven, Thompson, Nahum, & Haskins, 1990; Ryan, Vega, & Drash, 1985; Ryan & Williams, 1993) and the relative risk of developing AD is about 1.9 to 2.3 for diabetic patients (Leibson et al., 1997; Ott et al., 1996; Ronnemaa et al., 2009). Diabetes was shown to increase not only the risk of dementia but also the risk of progression from mild cognitive impairment to AD (Velayudhan et al., 2010). Uncontrolled diabetes (no insulin treatment) was associated with the development of AD, while patients with controlled diabetes showed no increased dementia, suggesting a role of impaired insulin signaling in the development of neurodegeneration and AD (Xu, von Strauss, Qiu, Winblad, & Fratiglioni, 2009). More recently, this hypothesis has been tested by several groups (Artola et al., 2002; Batista et al., 2018; Chiu, Chen, & Cline, 2008; Morris & Burns, 2012; Salameh et al., 2015). In addition to multiple possible mechanisms and pathways, the implication of the insulin signaling pathway (Gotz, Ittner, & Lim, 2009; Jolivalt et al., 2008; Jones, Kulozik, Ostertag, & Herzig, 2009) proved to be important to the convergence of both diabetes and AD, suggesting that treatments against diabetes that modulate insulin may prove efficacious in AD.

One of the current therapies for diabetes is the incretin-based treatments, particularly based on glucagon-like peptide 1 (GLP-1) (Lovshin & Drucker, 2009). GLP-1 is an endogenous insulinotropic peptide hormone. GLP-1 improves glycemic control in patients with type 2 diabetes, based on its abilities to enhance glucose-dependent insulin secretion from pancreatic β cells, to suppress glucagon secretion and to slow gastric emptying (for review Kieffer & Habener, 1999). GLP-1 has also been shown to improve markers of cognitive functions in type 2 diabetes models (Porter, Faivre, Flatt, Holscher, & Gault, 2012). In addition, GLP-1 was shown to be effective in newly diagnosed type 1 diabetic patients, with residual β cells mass (Behme, Dupre, & McDonald, 2003; Dupre, Behme, & McDonald, 2004; Kielgast, Holst, & Madsbad, 2009). In type 1 diabetic patients, GLP-1 also inhibits gastric emptying and glucagon secretion, and promotes satiety, therefore reducing blood glucose in patients suffering from type 1 diabetes, despite the lack of β cells (Creutzfeldt et al., 1996; Gutniak, Orskov, Holst, Ahren, & Efendic, 1992). A number of long-lasting analogs have been developed to treat type 2 diabetes, including liraglutide and exendin-4 (Ex4), commercialized as Byetta^{TM}. In addition to their insulinotropic activity, GLP-1 and Ex4 have been shown to have neurotrophic properties involving activation of the PI3K pathway in cultured neurons, in rats with pyridoxine-induced peripheral sensory neuropathy

Significance

Using in vivo mouse models with insulin deficiency or resistance and combination of diabetes and AD, we have demonstrated the role of insulin deficiency rather than insulin resistance in the development of cognitive dysfunction. In contrast to the HFD model of insulin resistance, the STZ mouse model of type 1 diabetes also displays hyperglycemia, which may also contribute to the deficits observed. In addition, Ex4 therapy with its neuroprotective properties and its effects on insulin and adiponectin may be a promising candidate drugs to halt cognitive deterioration in diabetes and AD patients.

(Perry et al., 2002, 2007), and on peripheral neuropathy in diabetic mice (Jolivalt, Fineman, Deacon, Carr, & Calcutt, 2011). GLP-1 receptors are widely distributed in rodent dorsal root ganglia and sciatic nerve, more particularly in axons and Schwann cells (Jolivalt et al., 2011) as well as in rodent and human brain (Perry et al., 2003). Ex4 rapidly crosses the blood: brain barrier and enters the brain where GLP-1R are present on neurons (Hamilton & Holscher, 2009). The GLP-1 effects in the brain are not limited to the regulation of appetite but also results in a variety of neurotrophic and neuroprotective actions (Erbil et al., 2019). GLP-1 was shown to reduce amyloid β levels in mouse brain (Perry et al., 2003) and in a model of AD (Li et al., 2010). In recent studies, GLP-1 or analogs were shown to ameliorate neurodegeneration, improve learning and memory in the Morris water maze and reversed icv-STZ-induced tau phosphorylation (Li et al., 2012) via down-regulation of glucogen synthase kinase 3 β activity (Chen, Liu, An, Yao, & Gao, 2012). Ex4 was also shown to improve cognitive behavior and neurogenesis in the hippocampus of adult rodents (Isacson et al., 2011) and in a mouse model of obesity and insulin resistance (Gault, Porter, Flatt, & Holscher, 2010). Similarly, in the STZ mouse model of type 1 diabetes, in which we have demonstrated AD-like features and insulin signaling impairment in the brain (Jolivalt et al., 2008), we have shown that Ex4 significantly prevented memory impairments in the Barnes maze after 8 weeks of treatment (unpublished data), without affecting plasma glucose or insulin levels (Jolivalt et al., 2011), suggesting that activation of GLP-1 receptor provides neuroprotective effect in the brain of type 1 diabetic mice independent of direct glucose or insulin modulation.

We have characterized and compared the effect of 12 weeks of insulin deficiency or insulin resistance on the progression of AD using an animal model of AD, the tau and amyloid precursor protein (TAPP) mouse, carrying the two major hallmarks of the disease (NFT and plaques), closely reflecting the human disease. Based on the link between AD and diabetes and the properties of Ex4, an intervention paradigm, mirroring the clinical setting when patients consult experiencing cognitive deficits, was applied to wild type (WT) and TAPP mice with concomittent type 1 diabetes, providing an option

to ameliorate the hastened development of AD in patients with type 1 diabetes.

2 | MATERIAL AND METHODS

2.1 | Animals

Female WT (mixed background C57Bl6, SJL, DBA/2, SW) mice and double transgenic mice (Tg(APPSWE)2576KhaTg(Prnp-MAPT*P301L)JNPL3Hlmc, abbreviated as TAPP) from Taconic, USA, were used in the studies. These TAPP mice carry the transgene coding for the 695-amino acid isoform of human Alzheimer β-amyloid (Aβ) protein in addition to the transgene for the human P301L mutation of the microtubule-associated protein tau (MAPT) gene. Double transgenic mice (mutant human APP and human tau: TAPP) develop few plaques and neurofibrillary tangles (NFT) in the limbic system by 6 months of age that become numerous by 8–9 months of age (Lewis et al., 2001). Mice were housed four to five per cage with free access to water and food. Studies followed protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

These mice were rendered diabetic (STZ or HFD) at 3–4 months of age and followed for 3 months to characterize the model (n = 9 per group based on power calculation, WT, WT-STZ, WT-HFD, TAPP, TAPP-STZ and TAPP-HFD groups). The therapeutical intervention study was starting after 5–6 weeks of untreated diabetes followed by 6 weeks of treatment with Ex4 at 10 μg/kg sc 2x/day (n = 12, based on power calculation with additional mice to account for possible death due to type 1 diabetes, WT, WT-STZ, WT-STZ-Ex4, TAPP, TAPP-STZ, TAPP-STZ-Ex4 groups).

2.2 | Drugs

Ex4 was purchased from American Peptide Company, (Sunnyvale, USA) and dissolved in 0.9% sterile saline. Streptozotocin (STZ) was purchased from Sigma (USA) and dissolved in 0.9% sterile saline. Unless indicated otherwise, assays were performed 24 hr after the most recent injection to obviate any acute effects of treatment.

2.3 | Insulin-deficient type 1 diabetes

Type 1 diabetes was induced by injection of streptozotocin (STZ: 90 mg/kg, Sigma, St. Louis, USA) that was freshly dissolved in 0.9% sterile saline and administered on two consecutive days, each preceded by an overnight fast. Hyperglycemia was measured using a test strip and meter (One Touch Ultra, Lifescan Inc. Milpitas, USA) in a blood sample obtained by tail prick 4 days after the second STZ injection and at regular intervals thereafter. Mice with a blood glucose level above 270 mg/dl were accepted as diabetic. This regime

does not produce direct STZ-induced neurotoxicity (Davidson et al., 2009).

2.4 | Obesity-induced insulin resistance

Mice were fed a standard diet containing only 10% fat (Low-fat diet [LFD], 10% kcal from fat, Research Diet Inc., New Brunswick, NJ, USA) for the control and STZ groups or a high-fat diet (HFD, 60% kcal from fat, Research Diet Inc., New Brunswick, NJ, USA) for obesity-induced insulin resistance groups.

2.5 | Glucose tolerance test

To assess insulin resistance, glucose was administered at 1.5 g/kg ip after on overnight fast. Glucose levels were measured using a strip-operated reflectance meter in a blood sample obtained by tail prick every 30 min for 2 hr.

2.6 | Barnes circular maze task

Learning and memory abilities were assessed using the Barnes maze test as described previously (Jolivalt et al., 2008, 2010). Briefly, the Barnes circular maze consists of an illuminated white circular platform with 20 holes (5 cm diameter) equally spaced and located 5 cm from the perimeter. A black escape box was placed under one of the holes for the learning phase of the test. A cue was placed behind the hole with the escape box. The mouse was placed in the middle of the platform and allowed to explore the maze. Timing of the session ended when the mouse found the box or after 5 min had elapsed. The memory phase of the test consisted of placing the mouse back on the Barnes maze, where the escape box was removed but the visible cue maintained, after 3 days without exposure to the maze. Time to find the box (during the learning phase) or the location of the box (during the memory phase) and number of errors (picking in the holes without the escape box) were recorded. Mice were tested after 8 and 12 weeks of untreated insulin deficiency and resistance and after 6 weeks of untreated STZ diabetes followed by 6 additional weeks of treatment with vehicle or Ex4, once a day, for 4 or 5 consecutive days for the learning phase and after 3 days without testing at day 9 for the memory phase of the test.

2.7 | Plasma analysis

Plasma obtained at termination of the study was assessed for insulin (Ultrasensitive mouse insulin ELISA, Mercodia AB, Uppsala, Sweden), adiponectin (HMW and total adiponectin ELISA, Alpco Immunoassays, Salem, NH, USA) and for triglycerides levels using

the oxygen-rate analyzer GM7 (Analox Instruments, Lunenburg, MA, USA).

2.8 | Western blotting

Hippocampi were homogenized in buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X, 1 mM EDTA, protease inhibitor cocktail). Fifty microliter of the homogenate was boiled for 5min, iced for 5min and then centrifuged to purified the preparation as tau proteins are heat resistant. Protein concentration was assessed using the bicinchoninic acid method (BCA protein assay kit, Pierce, Rockford, IL, USA). Homogenates (20 μg) and boiled homogenates (5 μg) were then separated on 4%–12% SDS-PAGE Bis-Tris gels (Novex, Invitrogen, Carlsbad, CA, USA) and immunoblotted onto nitrocellulose as described previously (Jolivalt et al., 2008). To maximize the number of proteins analyzed by Western blot, membranes were cut along the molecular weight markers into strips containing the proteins of interest. Blot strips were incubated with antibodies (Table 1) against phosphorylated tau (T231, 1/3,000,Chemicon, cat# AB9668, RRID:AB_570891), tau (1/3,000, Chemicon, cat#MAB361, RRID:AB_94944), amyloid β (1/1,000, Covance, cat# SIG-39300, RRID:AB_662807), or actin (1/1,000, Sigma, cat# A4700, RRID:AB_476730) followed by the corresponding secondary antibodies tagged with infrared dyes (IRDye, 1/15,000, LI-COR Biosciences, Lincoln, NE, USA). For each animal, band intensities were normalized by calculating the ratio of the intensity of the band corresponding to the primary antigen of interest to the intensity of the band corresponding to total tau for tau phosphorylation and to actin for amyloid β. Two or three Western blots were necessary to analyze all the samples for each study. These two to three blots were replicated at least three times and intensity values were averaged to provide the percent intensities reported.

2.9 | Analysis

All animals and tissues were coded during assay to prevent observer bias. Data are presented as group mean $\pm$ SD. Outliers were removed when larger than 2xSD. Using GraphPad Prism (version 8.0), statistical comparisons were made using mixed-effects analysis (REML) followed by Dunnett's post hoc test or by one-way ANOVA with differences identified compared with the appropriate controls using Sidak's post hoc test.

3 | RESULTS

3.1 | Characterization of the models

3.1.1 | Physiology

TAPP mice had similar body weight than aged-matched WT mice over the 12 weeks of the study. Insulin-deficient diabetes in STZ-injected mice resulted in a slight weigh loss in both WT and TAPP mice that was then maintained for the rest of the study while 12 weeks on HFD induced a similar weigh gain for both strains of mice (Figure 1a). At the end of the study, blood glucose levels significantly ($F_{(5,41)} = 44.66$, $n = 43$, $p < 0.0001$) increased in WT and TAPP with insulin-deficient diabetes or type 1 diabetes (Table 2). This was confirmed after 12 weeks of diabetes with significantly ($F_{(5,38)} = 269.4$, $n = 43$, $p < 0.0001$) increased HbA1c, an indicator of prolong hyperglycemia. In contrast, blood glucose and HbA1c levels were not significantly increased in both WT and TAPP mice on HFD (Table 2). However, 12 weeks on HFD induced insulin resistance in both WT and TAPP mice as indicated by the glucose tolerance test (Figure 1b). In parallel, insulin levels were reduced in STZ mice while increased in HFD mice for both strains of mice (Table 2). Although WT and TAPP basal insulin levels were similar, 12 weeks on HFD induced a significantly ($F_{(5,37)} = 14.27$, $n = 43$, $p = 0.0001$) higher increase in insulin levels in TAPP mice compared with WT mice (TAPP-HFD: 5.09 $\pm$ 0.92 vs. WT-HFD:1.42 $\pm$ 0.36 μg/l). Inversely, triglycerides levels were significantly ($F_{(5,34)} = 5.32$, $n = 40$, $p = 0.0010$) increased in STZ mice but not in HFD mice for both strains of mice (Table 2). Plasma adiponectin levels were reduced in TAPP mice compared with WT mice. Insulin-deficient diabetes (STZ) significantly reduced ($F_{(5,38)} = 8.27$, $n = 43$, $p < 0.0001$) adiponectin levels in WT (Table 2).

TABLE 1 Antibodies used

| Name	Target/Immunogen	Species/ Poly-monoclonal	Manufacturer Cat #	RRID	Dilution
Actin	Clone AC40	Mouse Monoclonal	Sigma A4700	AB_476730	1/1,000
Amyloid β	Amyloid 1-40/42	Mouse Monoclonal	Covance SIG-39300	AB_662807	1/1,000
pTau	phosphorylated tau at Thr 231	Mouse Monoclonal	Chemicon MAB361	AB_94944	1/3,000
Tau	Tau aa 210-241	Rabbit Polyclonal	Chemicon AB9668	AB_570891	1/3,000
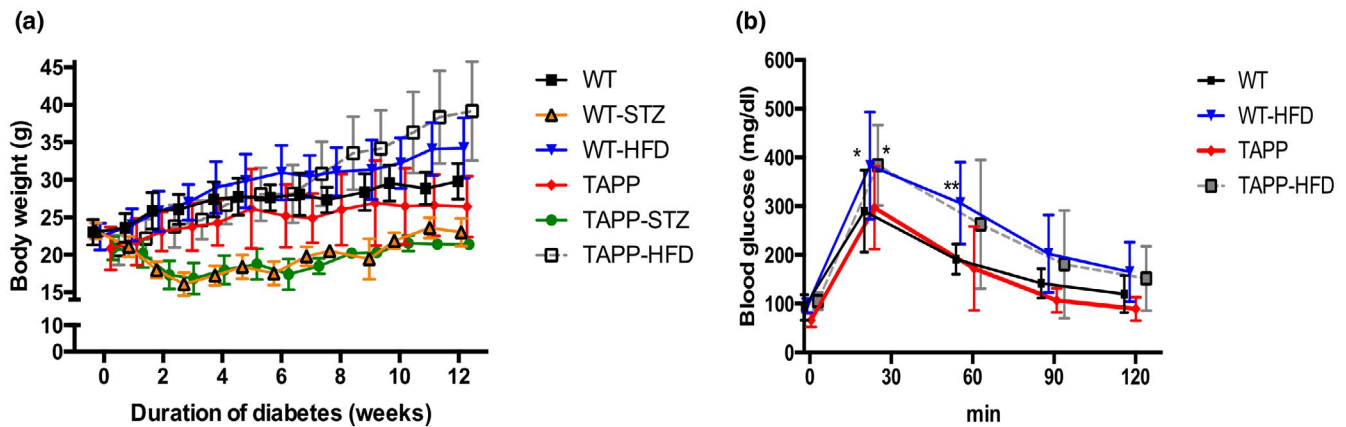
FIGURE 1 Physiology. (a) Weight gain during the course of the study. (b) Blood glucose after a glucose challenge at the end of the study. Mean ± SD, $n = 5$–9 $*p < 0.05$, $**p < 0.01$, compared with WT by mixed-effects analysis followed by Dunnett's post hoc test. Groups: Wild type (WT, black square), WT-STZ (upward triangle orange line), WT-HFD (downward blue triangle), TAPP (red diamond), TAPP-STZ (green circle) and TAPP-HFD (open square dotted gray line)

TABLE 2 Physiological parameters at the end of the study

Groups	Blood glucose (mg/ml)	HbA1c (%)	Insulin (µg/l)	Triglycerides (mmol/l)	Adiponectin (µg/ml)
WT	138.6 ± 24.9	4.23 ± 0.14	0.79 ± 0.26	1.17 ± 0.34	12.59 ± 2.72
WT-STZ	544.8 ± 82.6***	12.80 ± 0.45***	0.22 ± 0.14	2.96 ± 1.77**	6.85 ± 1.98**
WT-HFD	139.2 ± 23.3	4.20 ± 0.19	1.42 ± 1.02	1.16 ± 0.18	10.54 ± 5.57
TAPP	135.6 ± 20.5	4.20 ± 0.18	0.79 ± 0.42	1.34 ± 0.41	7.77 ± 1.78
TAPP-STZ	528.7 ± 66.9###	11.88 ± 1.66###	0.38 ± 0.22	2.97 ± 1.69##	4.44 ± 1.34
TAPP-HFD	163.9 ± 48.1	4.49 ± 0.47	5.09 ± 2.76###	1.72 ± 0.50	5.09 ± 2.44

Note: Mean ± SD, $n = 5$–9 $**p < 0.01$, $***p < 0.001$, compared with WT and ##$p < 0.01$, ###$p < 0.001$ compared with TAPP by one-way ANOVA followed by Sidak's post hoc test.

3.1.2 | Learning and memory behavior

After 8 weeks of insulin deficiency and insulin resistance, WT and TAPP mice learned the Barnes maze task of finding the escape box in a similar fashion ($F_{(5,42)} = 0.07$, $n = 48$, $p = 0.9957$, without interaction $F_{(15,126)} = 1.39$, $p = 0.1581$) (Figure 2a). After 12 weeks of insulin impairments, WT-STZ, TAPP, TAPP-STZ, and TAPP-HFD mice take longer time, although not significantly different, to find the escape box than WT mice, indicative of marginal learning impairments ($F_{(5,41)} = 2.62$, $n = 47$, $p = 0.0378$, without significant interaction $F_{(15,123)} = 1.32$, $p = 0.1966$) (Figure 2b,c) despite similar abilities on the rotarod (Time on the rod in seconds, mean ± SD, WT:19.2 ± 12.7, WT-STZ: 28.8 ± 18.8, WT-HFD:12.9 ± 7.8, TAPP:23.44 ± 18.2, TAPP-STZ = 14.7 ± 4.2, TAPP-HFD: 10.8 ± 5.6). WT mice on HFD did not show learning impairments (Figure 2b,c).

Memory was assessed after 3 days without exposure to the maze and was significantly ($F_{(5,41)} = 3.09$, $n = 47$, $p = 0.0018$) impaired in TAPP-STZ mice after 8 weeks of diabetes (Figure 2d). By 12 weeks of diabetes, both WT and TAPP mice with insulin-deficient diabetes (STZ) displayed impaired memory in the Barnes maze while mice with insulin resistance did not ($F_{(5,39)} = 3.51$, $n = 40$, $p = 0.0103$) (Figure 2e).

3.1.3 | AD hallmarks

Hippocampi were collected, homogenized, and submitted to Western blot analysis for phosphorylated tau and soluble amyloid β proteins. Tau phosphorylation was significantly ($F_{(5,38)} = 4.35$, $n = 43$, $p = 0.0013$) increased in insulin-deficient mice when compared with their respective controls (Figure 3a). Insulin resistance did not affect tau phosphorylation levels. A soluble oligomer of Aβ (24 kDa) was significantly ($F_{(5,27)} = 20.21$, $n = 33$, $p < 0.0001$) increased in TAPP mice hippocampus compared with WT mice (Figure 3b). Amyloid β protein levels were increased by insulin-deficient diabetes but not by insulin resistance in both WT and TAPP mice brain (Figure 3b).

3.2 | Exenatide treatment

Given the results described above, we have selected insulin-deficient diabetes in WT and TAPP mice in order to assess the effect of a long-lasting analog of GLP-1, Ex4. Ex4 treatment was initiated after 6 weeks of untreated type 1 diabetes to reflect the more likely scenario when
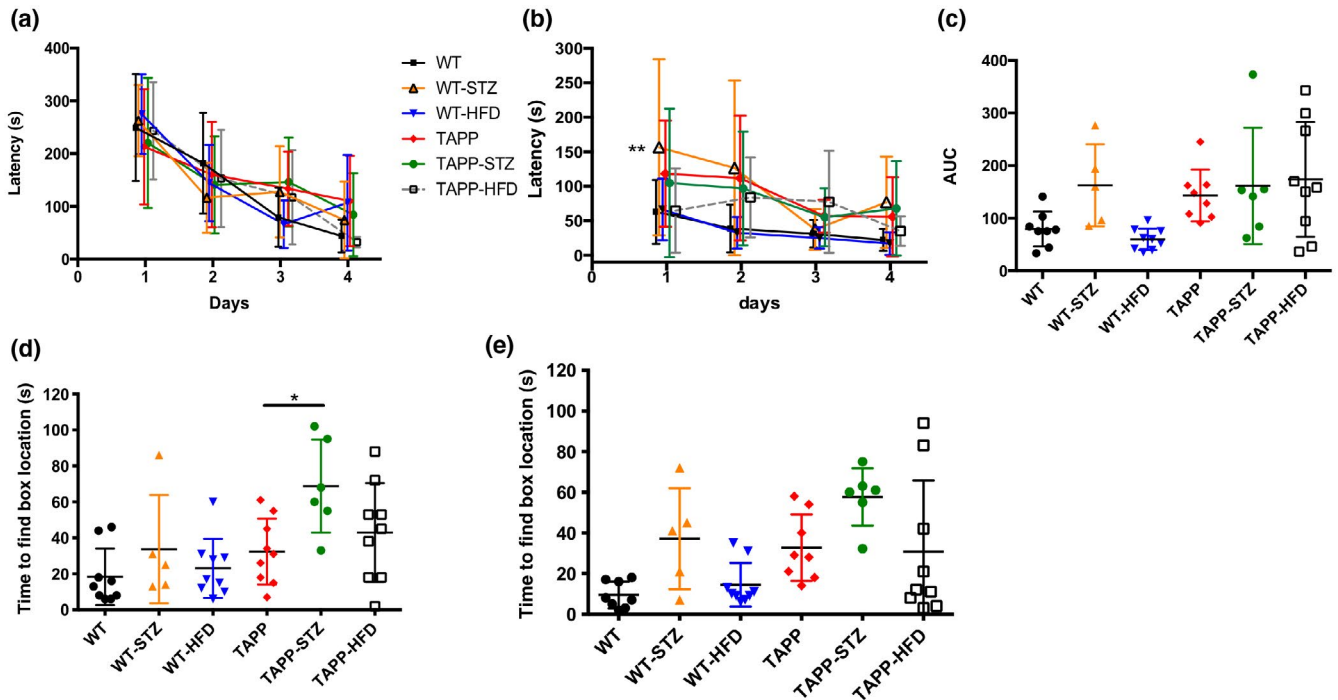
FIGURE 2 Cognitive performance. (a) Latencies to find the escape box in the Barnes maze at 8 weeks of diabetes. (b) Latencies to find the escape box in the Barnes maze at 12 weeks of diabetes. $**p < 0.01$ compared with WT by mixed-effects analysis followed by Dunnett's post hoc test. Groups: Wild type (WT, black square), WT-STZ (upward triangle orange line), WT-HFD (downward blue triangle), TAPP (red diamond), TAPP-STZ (green circle), and TAPP-HFD (open square dotted gray line). (c) Area under the curve (AUC) of graph B. (d) Memory retention in the Barnes maze at 8 weeks of diabetes. (e) Memory retention in the Barnes maze at 12 weeks of diabetes. Mean $\pm$ SD, $n = 5$–9 $*p < 0.05$, $**p < 0.01$, by one-way ANOVA followed by Sidak's post hoc test. Groups: Wild type (WT, black circle), WT-STZ (upward orange triangle), WT-HFD (downward blue triangle), TAPP (red diamond), TAPP-STZ (green circle), and TAPP-HFD (open square)
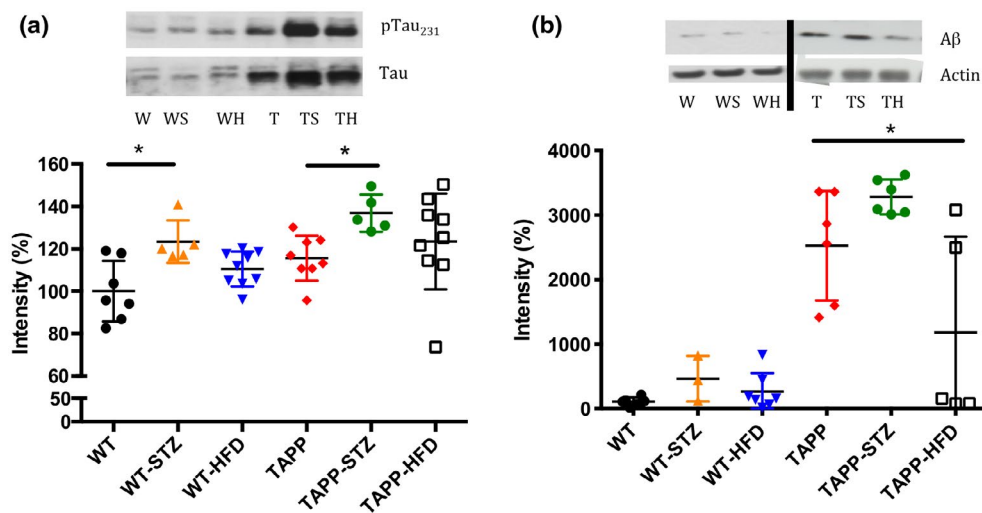

FIGURE 3 Hippocampal protein levels. (a) Levels of phosphorylated tau at Threonine 231 in mouse hippocampus. (b) Soluble amyloid β (24 kDa oligomer) levels in mouse hippocampus. W: Wild type (WT), WS: WT-STZ, WH: WT-HFD, T: TAPP, TS: TAPP-STZ, TH: TAPP-HFD. Mean $\pm$ SD, $n = 5$–9 $*p < 0.05$, $**p < 0.01$, by one-way ANOVA followed by Sidak's post hoc test. Groups: Wild type (WT, black circle), WT-STZ (upward orange triangle), WT-HFD (downward blue triangle), TAPP (red diamond), TAPP-STZ (green circle), and TAPP-HFD (open square)

patients with diabetes and noticeable cognitive impairments will consult their physician.

3.2.1 | Physiology

After 12 weeks of diabetes, including the last 6 weeks of treatment with vehicle or Ex4, insulin-deficient diabetes induced a significant ($F_{(5,61)} = 5.27$, $n = 67$, $p = 0.0004$) weight loss in TAPP mice that was not corrected by treatment with Ex4 (WT: 28.4 ± 3.5, WT-STZ:27.7 ± 2.5, WT-STZ-Ex4:27.2 ± 2.4, TAPP:28.4 ± 2.0*, TAPP-STZ: 24.6 ± 2.4, TAPP-STZ-Ex4: 24.7 ± 2.9, *$p < 0.05$ compared with TAPP-STZ by one-way ANOVA followed by Sidak's post hoc test). Final blood glucose levels were significantly ($F_{(5,59)} = 140.6$, $n = 65$, $p < 0.0001$) elevated in STZ-diabetic mice for both WT and TAPP mice (Table 3). Only in TAPP mice, blood glucose levels were significantly reduced, but remained elevated, by treatment with Ex4, (Table 3). HbA1c levels after 12 weeks of diabetes were significantly ($F_{(5,61)} = 38.08$, $n = 67$, $p < 0.0001$) elevated in all STZ mice treated with vehicle or Ex4 (Table 3). Treatment with Ex4 for 6 weeks significantly increased insulin levels to levels above their respective controls in both WT and TAPP mice (Table 3). Triglycerides levels were significantly ($F_{(5,59)} = 8.17$, $n = 64$, $p < 0.0001$) elevated in diabetic WT and TAPP mice and 6 weeks of treatment with Ex4 tend to reduce these levels in both strains of mice (Table 3). Type 1 diabetes for 12 weeks induced a significant ($F_{(5,55)} = 6.79$, $n = 61$, $p < 0.0001$) decreased of plasma adiponectin levels in WT and TAPP mice and 6 weeks of treatment with Ex4 significantly elevated these levels in TAPP-STZ mice (Table 3).

3.2.2 | Learning and memory behavior

In the Barnes maze test, at 5–6 months of age and 6 weeks of untreated diabetes, TAPP mice developed significant ($F_{(5,63)} = 2.35$, $n = 69$, $p = 0.0505$, without significant interaction: $F_{(15,189)} = 1.25$, $p = 0.2359$) impairment in learning abilities compared with age-matched WT (Figure 4a). After 6 weeks of untreated insulin-deficient diabetes, before Ex4 treatment started, WT-STZ and TAPP-STZ mice showed similar learning impairments (Figure 4a). After 12 weeks of untreated diabetes, WT-STZ and TAPP-STZ

displayed a worsening of learning abilities compared with their respective controls ($F_{(5,65)} = 6.55$, $n = 71$, $p < 0.0001$, with significant interaction $F_{(20,260)} = 1.79$, $p = 0.0218$) (Figure 4b), despite similar deambulation and picking in holes without the escape box (error)(% error, mean $\pm$ SD, WT: 5.5 ± 9.0, WT-STZ: 2.6 ± 4.7, WT-STZ-Ex4: 5.5 ± 6.4, TAPP: 15.5 ± 17.7, TAPP-STZ: 10.5 ± 11.1, TAPP-STZ-Ex4: 10.1 ± 12.0) and 6 weeks of treatment with Ex4 significantly ($F_{(5,57)} = 7.83$, $n = 63$, $p < 0.0001$) prevented the cognitive worsening (Figure 4b,c). Memory, as shown above (Figure 2e), was impaired in WT-STZ and TAPP-STZ mice after 12 weeks of untreated diabetes, however, 6 weeks of intervention with Ex4 did not ameliorate the memory function in either strain of mice (Figure 4d).

3.2.3 | AD hallmarks

Tau phosphorylation was significantly ($F_{(5,57)} = 4.31$, $n = 63$, $p = 0.0036$) increased in the hippocampus of TAPP mice with concurrent type 1 diabetes and tend to decrease after 6 weeks of treatment with Ex4 (Figure 5a). Soluble amyloid β oligomer (24 kDa) levels were significantly increased in TAPP mice, further increased with concomitant diabetes and significantly ($F_{(5,59)} = 14.34$, $n = 65$, $p < 0.0001$) reduced by intervention with Ex4 (Figure 5b). To ensure that actin levels were constant between groups, we normalized bands corresponding to actin protein against cyclophillin B protein bands. No difference among group was found (band intensity as mean $\pm$ SD, WT: 100.0 ± 9.4, WT-STZ: 98.7 ± 13.6. WT-STZ-Ex4: 96.6 ± 14.7, TAPP: 97.5 ± 15.7, TAPP-STZ:99.0 ± 14.2, TAPP-STZ-Ex4: 100.0 ± 9.4). Neuronal loss is another major hallmark of AD. Here, we assessed the neuronal loss using NeuN immunostaining of the hippocampus. NeuN immunostaining was significantly ($F_{(5,57)} = 4.63$, $n = 63$, $p = 0.0005$) reduced by type 1 diabetes in both strains of mice (Figure 5c). Six weeks of intervention with Ex4 after 6 weeks of untreated diabetes did significantly improve the percent of NeuN positive cells in TAPP mouse hippocampus (Figure 5c).

4 | DISCUSSION

Insulin deficiency, but not insulin resistance is sufficient to induce learning and memory impairments with accumulation of

TABLE 3 Effect of Exendin-4 treatment on mouse physiology

Groups	Blood glucose (mg/ml)	HbA1c (%)	Insulin (µg/l)	Triglycerides (mmol/l)	Adiponectin (µg/ml)
WT	151.0 ± 33.2***	4.69 ± 0.42***	0.89 ± 0.52	2.46 ± 1.15***	4.63 ± 2.31***
WT-STZ	572.7 ± 44.5	10.08 ± 1.52	0.60 ± 0.15	6.60 ± 3.42	1.92 ± 1.03
WT-STZ-Ex4	538.0 ± 53.7	9.76 ± 2.05	4.35 ± 2.91***	4.53 ± 2.63	3.11 ± 1.05
TAPP	184.6 ± 42.0###	4.38 ± 0.38###	1.09 ± 0.33	1.56 ± 0.62##	3.35 ± 1.09#
TAPP-STZ	585.9 ± 21.2	8.29 ± 1.19	0.64 ± 0.16	4.72 ± 1.76	1.25 ± 0.42
TAPP-STZ-Ex4	513.2 ± 88.3##	8.50 ± 1.29	2.22 ± 1.75#	3.37 ± 1.78	2.77 ± 1.06#

Note: Mean $\pm$ SD, $n = 9$–13 ***$p < 0.001$ compared with WT-STZ and #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared with TAPP-STZ by one-way ANOVA followed by Sidak's post hoc test.
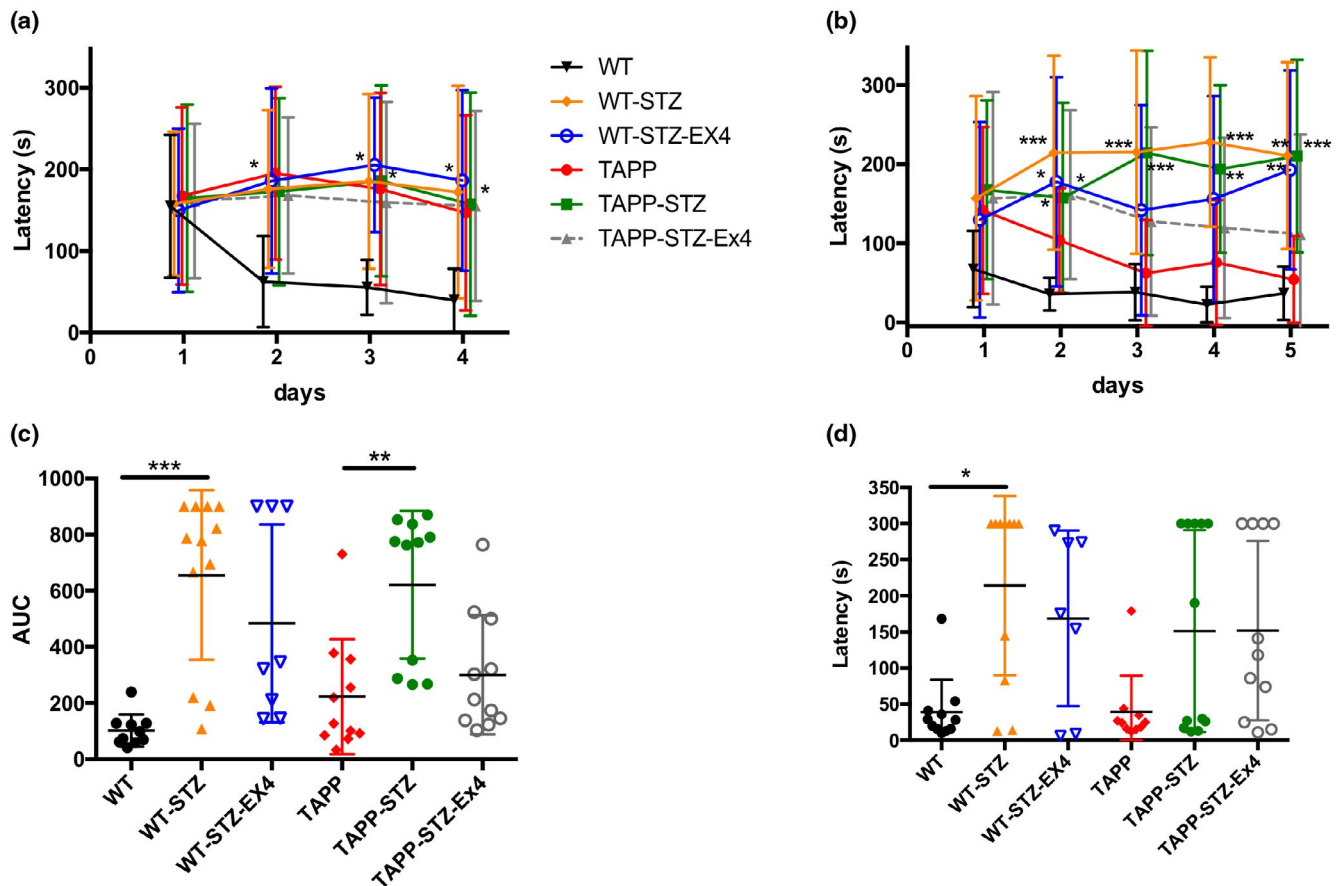
FIGURE 4 Effect of Exendin-4 on learning and memory. (a) Latencies to find the escape box in the Barnes maze at baseline after 6 weeks of diabetes. (b) Latencies to find the escape box in the Barnes maze after 12 weeks of diabetes, including the last 6 weeks of treatment. Groups: Wild type (WT, black downward triangle), WT-STZ (orange diamond), WT-STZ-Ex4 (open blue circle), TAPP (red circle), TAPP-STZ (green square) and TAPP-STZ-EX4 (upward gray triangle). (c) Area under the curve (AUC) of graph B. (d) Memory retention in the Barnes maze after 12 weeks of diabetes, including the last 6 weeks of treatment. Mean $\pm$ SD, n = 7–12 *p < 0.05, **p < 0.01, ***p < 0.001 compared with WT by mixed-effects analysis followed by Dunnett's post hoc test. Groups: Wild type (WT, black circle), WT-STZ (upward orange triangle), WT-STZ-Ex4 (downward open blue triangle), TAPP (red diamond), TAPP-STZ (green circle), and TAPP-STZ-Ex4 (open circle)

phosphorylated tau and soluble amyloid β oligomers in the brain as previously shown by us and others (Jolivalt et al., 2008; Kim, Backus, Oh, Hayes, & Feldman, 2009). Consistent with our previous studies (Jolivalt et al., 2010), diabetes exaggerates AD progression in a model of AD carrying both human tau and APP genes. Again, only type 1 insulin-deficient diabetes affected memory, neuronal loss, amyloid oligomers, and phosphorylated tau accumulation in the brain of TAPP mice. Insulin resistance did not significantly affect these hallmark parameters in the time frame studied. Furthermore, the exaggeration of AD-like features by type 1 diabetes was partially halted by treatment with Ex4.

4.1 | Insulin deficiency but not insulin resistance exaggerates deficits

We have already shown increased Aβ and phosphorylated tau levels in STZ mice hippocampus (Jolivalt et al., 2008), confirmed in STZ rats (Liu et al., 2008) and that insulin-deficient diabetes exaggerates

the development of AD-like features in the brain of transgenic mice overexpressing only APP (Jolivalt et al., 2010). In a mouse model of AD with both major proteins overexpressed leading to Aβ and phosphorylated tau deposition, the direct comparison between insulin deficiency and insulin resistance for the same duration demonstrated that in the time frame studied only insulin deficiency, albeit with concomitant hyperglycemia, affects all the parameters measured. This contrasts with studies showing memory impairments in HFD fed mice (Ayabe, Ohya, & Ano, 2019; Sah, Lee, Jang, & Park, 2017; Sanguinetti et al., 2019) possibly due to different mouse backgrounds, HFD composition and/or duration of study. However, in line with our results, HFD did not induce overall cognitive dysfunction in the Barnes maze in adult mice (Kesby et al., 2015). In addition, Petrov et al (Petrov et al., 2015) showed that phosphorylated tau levels were not increased and alterations in cerebral amyloid levels did not play a critical role in memory loss of APP-PS1 transgenic mice when fed HFD from weaning for 6 months. More recently, consistent with our findings, a similar lack of elevated Aβ and phosphorylated tau levels in triple transgenic AD mice were found after 6 months of
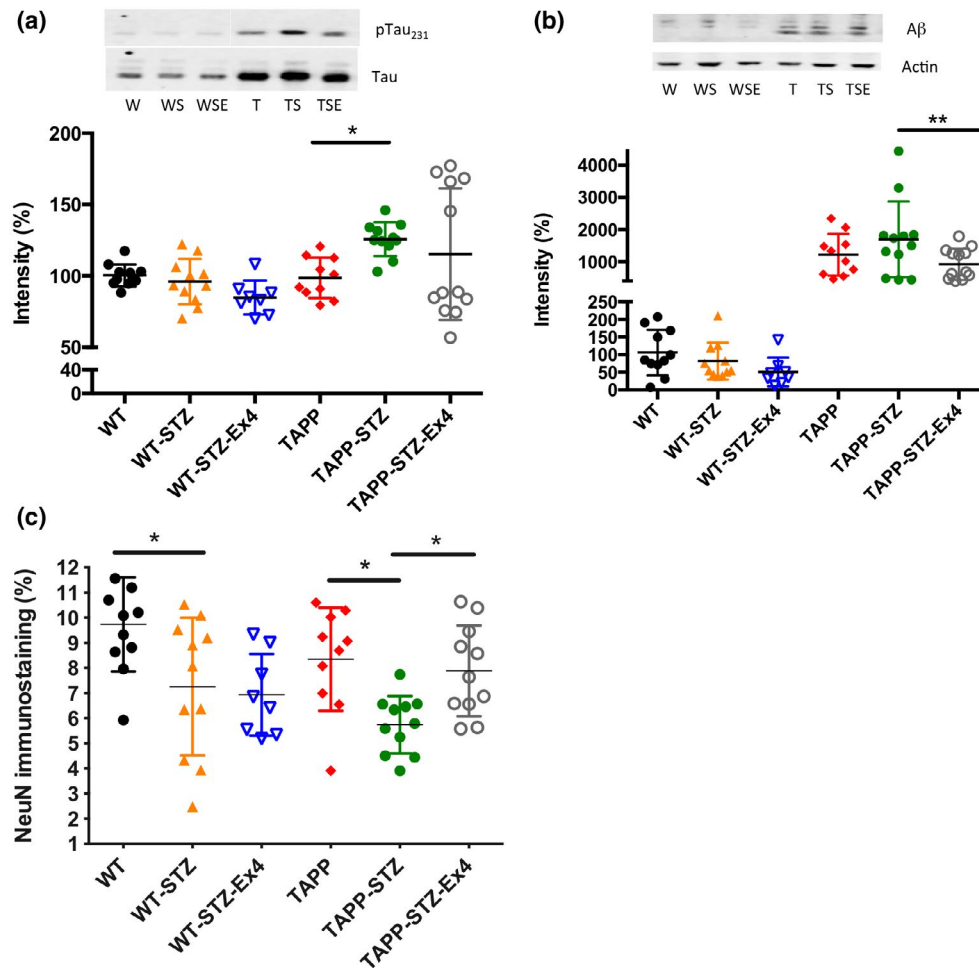
FIGURE 5 Effect of Exendin-4 on protein levels and neuronal loss. (a) Levels of phosphorylated tau at Threonine 231 in mouse hippocampus after 12 weeks of diabetes, including 6 weeks of treatment with Exendin-4. (b) Soluble amyloid β (24 kDa oligomer) levels in mouse hippocampus after 12 weeks of diabetes, including 6 weeks of treatment with Exendin-4. W: Wild type (WT), WS: WT-STZ, WSE: WT-STZ-EX4, T: TAPP, TS: TAPP-STZ, TSE: TAPP-STZ-Ex4. (c) NeuN intensity in the hippocampus of mouse after 12 weeks of diabetes, including 6 weeks of treatment with Exendin-4 or vehicle. Mean $\pm$ SD, $n = 8$–12 $^*p < 0.05$, $^{**}p < 0.01$, by one-way ANOVA followed by Sidak's post hoc test. Groups: Wild type (WT, black circle), WT-STZ (upward orange triangle), WT-STZ-Ex4 (downward open blue triangle), TAPP (red diamond), TAPP-STZ (green circle), and TAPP-STZ-Ex4 (open circle)

HFD in older mice and this was associated with no cognitive deficits (Bomba et al., 2019). Furthermore, our data are in agreement with the clinical data. The risk of developing dementia is increased by 65% in patients with type 1 diabetes versus 37% for patients with type 2 diabetes (Smolina, Wotton, & Goldacre, 2015). And despite the increased risk of developing AD in patients with type 2 diabetes (Gudala, Bansal, Schifano, & Bhansali, 2013), post-mortem studies of brain showed that type 2 diabetes is associated with a decrease and not an increased burden of amyloid plaques and tangles (Nelson et al., 2009), suggesting that type 2 diabetes does not accelerate the development of AD-like pathology in patients.

4.2 | Lipidemia and adiponectin

Another major difference between insulin deficiency and insulin resistance conditions is the variation of plasma triglycerides and adiponectin levels. Triglycerides levels were significantly increased in STZ mice (both WT and TAPP) but not in mice fed a HFD for 3 months. Inversely, adiponectin levels were decreased in STZ but not in HFD mice. The basal levels of adiponectin in TAPP mice were reduced compared with WT, consistent with the low serum levels of adiponectin in AD patients (Kamogawa et al., 2010; Li et al., 2015). This lower plasma adiponectin concentration was further reduced by diabetes. Adiponectin is a fat-derived hormone that plays a role in improving insulin sensitivity in major insulin target organs (Ruan & Dong, 2016) and an important role in several metabolic pathways, notably lipoprotein metabolism (Christou & Kiortsis, 2013; Matsubara, Maruoka, & Katayose, 2002). Several studies have shown that lowering adiponectin in mice increased triglycerides in serum (Oku et al., 2007; Wanninger et al., 2012) and that lower serum triglycerides results from enhancement of lipoproteins catabolism induced by adiponectin (Lapointe et al., 2011). The relationship between triglycerides and adiponectin correlates with our findings of higher triglycerides and

lower adiponectin levels in WT and TAPP with concomitant insulin deficiency but not insulin resistance. Furthermore, it was shown that reduced amounts of subcutaneous fat and low levels of adiponectin were associated with development of mild cognitive impairments in Japanese men (Kamogawa et al., 2010), supporting our results of low adiponectin and exaggerated AD features in type 1 insulin-deficient diabetes with or without AD, but not with insulin resistance.

4.3 | Ex4 treatment

In accord with Li et al, (Li et al., 2010), Ex4 preventive treatment reduced blood glucose, phosphorylated Tau and Amyloid β levels in the brain of diabetic AD mice. Here in addition we showed that an intervention paradigm with Ex4, after 6 weeks of untreated diabetes, was effective in vivo on indices of neuronal loss (NeuN) and learning abilities. These results are supported by the demonstration that Ex4 increased neurogenesis in Parkinson disease mouse model (Bertilsson et al., 2008) and increased synaptic plasticity and long-term potentiation in normal brain slices exposed to Aβ (Gault & Holscher, 2008). Recently, Ex4 was shown to restore spatial memory and reduce AD-like histopathology in APP/PS1 mice (Bomfim et al., 2012) and APP mice (Robinson et al., 2019). In our study, the mechanism of action of Ex4 against the AD-like features (tau, neuronal loss, Aβ, learning impairment) was not directly related to effect on the insulin signaling pathway (no modulation of insulin signaling pathway protein levels or phosphorylation, data not shown). Additionally, Ex4 did not affect peripheral parameters of hyperglycemia. However, Ex4 did improve peripheral insulin levels, possibly by regeneration of few remaining β cells (Tourrel et al., 2002; Xu, Stoffers, Habener, & Bonner-Weir, 1999), which may contribute to its beneficial effect. Although, this was not the case in Tg2576 AD mice. While Ex4 treatment for 8 months did not significantly impact serum insulin levels, when Ex4 was combined with intranasal insulin, there was no additive or synergistic effect on Aβ levels and learning functions in Tg2576 mice (Robinson et al., 2019). In addition to the increased plasma insulin levels, as also observed in 3xTg AD mice (Li et al., 2010), Ex4 treatment significantly reversed diabetes-induced changes in adiponectin and triglycerides levels, supporting their inversed relationship (Christou & Kiortsis, 2013). Several studies have shown that Ex4 induced increased adiponectin levels, directly promotes adiponectin secretion in adipocytes and improves insulin sensitivity (Kim Chung et al., 2009; Li et al., 2008; Pastel et al., 2016; Wang et al., 2017). Moreover, improving adiponectin levels with Ex4 treatment correlated with improved learning ability, which is consistent with studies showing a role of adiponectin in cognitive impairments (Kim et al., 2017; Ng et al., 2016; Wang, Jo, & Song, 2019; Zhang et al., 2017). Altogether, reduction of adiponectin levels may play a significant role in the development of AD features that can be undermined by treatment with Ex4.

The side-by-side comparison of insulin deficiency and insulin resistance on the development of AD-like features in diabetic mouse models and the exaggeration of AD hallmarks in a model of AD underlines that insulin deficiency is more detrimental to the brain than insulin resistance. It may seem counterintuitive with the numerous reports of type 2 diabetes associated with the development of AD. One limitation of our study is the use of only female mice, a different conclusion may be reached with male mice. Further studies will be required. A major difference between the insulin-deficient mice and the insulin resistant ones is the glycemia level. We cannot exclude that hyperglycemia may be a contributing factor to the development of AD features and may be a reason we have not detected similar deficits in our HFD mice which displayed normoglycemia in contrast to type 2 diabetes. One other possible explanation is the duration of type 2 diabetes before patients develop cognitive deficits, possibly corresponding to the advanced stage of type 2 diabetes when β cells fail and their diabetes resemble more closely type 1 diabetes (Fonseca, 2009). Our study also showed that, despite being a first-line treatment for type 2 diabetes, the actions of Ex4 via its ability to modulate adiponectin levels may translate to a potentially beneficial treatment for type 1 diabetes and/or AD, even when cognitive deficits are already present.

DECLARATION OF TRANSPARENCY

The authors, reviewers and editors affirm that in accordance to the policies set by the *Journal of Neuroscience Research*, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

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