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GLP-1 Receptor Stimulation Reduces Amyloid-β Peptide Accumulation and Cytotoxicity in Cellular and Animal Models of Alzheimer's Disease

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Abstract

Type 2 (T2) diabetes mellitus (DM) has been associated with an increased incidence of neurodegenerative disorders, including Alzheimer's disease (AD). Several pathological features are shared between diabetes and AD, including dysfunctional insulin signaling and a dysregulation of glucose metabolism. It has therefore been suggested that not only may the two conditions share specific molecular mechanisms but also that agents with proven efficacy in one may be useful against the other. Hence, the present study characterized the effects of a clinically approved long-acting analogue, exendin-4 (Ex-4), of the endogenous insulin releasing incretin, glucagon-like peptide-1 (GLP-1), on stress-induced toxicity in neuronal cultures and on amyloid-β protein (Aβ) and tau levels in triple transgenic AD (3xTg-AD) mice with and without streptozocin (STZ)-induced diabetes. Ex-4 ameliorated the toxicity of Aβ and oxidative challenge in primary neuronal cultures and human SH-SY5Y cells in a concentration-dependent manner. When 11 to 12.5 month old female 3xTg AD mice were challenged with STZ or saline, and thereafter treated with a continuous subcutaneous infusion of Ex-4 or vehicle, Ex-4 ameliorated the diabetic effects of STZ in 3xTg-AD mice, elevating plasma insulin and lowering both plasma glucose and hemoglobin A1c (HbA1c) levels. Furthermore, brain levels of Aβ protein precursor and Aβ, which were elevated in STZ 3xTg-AD mice, were significantly reduced in Ex-4 treated mice. Brain tau levels were unaffected following STZ challenge, but showed a trend toward elevation that was absent following Ex-4 treatment. Together, these results suggest a potential value of Ex-4 in AD, particularly when associated with T2DM or glucose intolerance.

Keywords

3xTg-AD mice; Alzheimer's disease; amyloid-β peptide; amyloid-β protein precursor; dementia; diabetes; extendin-4; glucagon-like peptide-1; neuroprotection; streptozocin; tau

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD) are chronic, age-related degenerative disorders that are leading causes of morbidity and mortality in the elderly. Each has attained epidemic proportion, affecting an excess of 171 [1] and 24 million people [2], respectively, worldwide. A variety of risk factors implicated in the development of T2DM, such as genetic predisposition, age, oxidative stress, obesity, diet, and physical inactivity [3, 4] appear to also be involved in AD, the most common form of senile dementia [5,6]. Consistently, a number of well-designed epidemiological studies have established a link between these two diseases [6–10], implicating T2DM as a risk factor for developing AD. The pancreas and brain are both highly insulin sensitive tissues. Moreover, both AD and T2DM share several clinical and biochemical features, particularly important amongst these is an impaired insulin signaling, suggesting overlapping pathogenic mechanisms [3,7,10-12]. Hence, an effective treatment strategy against T2DM could have potential value in AD.

Additionally, both AD and T2DM are conformational diseases that are characterized by deposits of insoluble protein aggregates. Within the AD brain, the amyloid-β protein (Aβ), and the microtubule-associated protein, tau, undergo typical changes in tertiary structure followed by their self-aggregation and deposition [13,14]. Aβ is a major component of extracellular amyloid plaques and a cleavage product of amyloid-β protein precursor (AβPP), a widely expressed type 1 integral membrane glycoprotein with a large extracellular N-terminal domain, a single transmembrane domain and a short cytoplasmic tail [13-15]. AβPP is sequentially cleaved by two aspartyl proteases, β- and γ-secretase, to yield a family of Aβ peptides of 40- and 42 amino acids that aggregate to form oligomeric species capable of inducing synaptic dysfunction and neuronal degeneration [13-15]. Hyperphosphorylated tau is a primary constituent of intracellular neurofibrillary tangles (NFTs) that, together with amyloid plaques, are distinguishing hallmarks of AD [13-16]. Interestingly, disease progression in T2DM, characterized by progressive insulin resistance of target tissues leading to progressively elevated plasma glucose levels, is also accompanied by deposition of the amyloidogenic protein amylin in insulin-producing pancreatic β-cells [17]. Mature 37 amino acid amylin is generated by proteolysis from an inactive 67 amino acid propeptide and can induce β-cell dysfunction and death [18].

A recent effective treatment strategy in T2DM is the use of incretin-based therapies based on the actions of the physiological peptide, glucagon-like peptide-1 (GLP-1) on its receptor (GLP-1R), utilizing the long-acting analog exendin-4 (Ex-4) [18]. GLP-1R agonists acutely act on β-cells to stimulate insulin gene transcription and glucose-dependent insulin release. Chronic actions include stimulation of β-cell proliferation, induction of islet neogenesis, and inhibition of β-cell apoptosis that, together, promote expansion of β-cell mass and the normalization of insulin signaling [18,19]. Ex-4 has been reported to readily cross the blood-brain barrier and enter the brain [20], where GLP-1R is expressed widely [21,22] and its activation results in multiple biological responses [21,23]. GLP-1R stimulation in brain is classically allied to regulation of appetite and satiety [18,21]. More recently, however, it has been associated with neurotrophic [21,24] and neuroprotective actions in both cellular and *in vivo* models of acute and chronic neurodegenerative conditions [25], including stroke [23], AD [25,26], Parkinson's disease (PD) [23,27,28], and Huntington's disease [29]. GLP-1 and Ex-4 have been reported to acutely reduce brain levels of Aβ following their intracerebroventricular administration to mice [26], to impact memory [30,31], and modulate synaptic plasticity [32]. Each of these actions is relevant to AD and may additionally be impacted by T2DM. We therefore characterized the actions of chronic GLP-1R stimulation, achieved by steady-state subcutaneous administration of Ex-4, in triple transgenic AD (3xTg-AD) mice known to develop amyloid plaques and NFTs [33], with and without streptozocin (STZ)-induced diabetes. In addition, to differentiate primary from secondary actions, the effect of Aβ and

oxidative stress were characterized in neuronal cultures in the presence and absence of GLP-1R stimulation, and the synthesis of Aβ was measured in the absence and presence of Ex-4 under conditions equivalent to euglycemia and hyperglycemia. The results of these studies suggest that Ex-4, an efficacious treatment for T2DM, may also be of therapeutic value in AD alone or in association with T2DM.

MATERIALS AND METHODS

Peptides Ex-4, the GLP-1R antagonist, Ex-9-39, and $A\beta_{1-42}$ were obtained from Bachem (Torrance, CA). Hydrogen peroxide and all other reagents were acquired from Sigma (St Louis, MO) unless otherwise stated.

Cell culture studies

Cell cultures—SH-SY5Y cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and were grown in a 1:1 mixture of Eagle's Minimum Essential Medium and Ham's F12 Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA). They were maintained at 37°C in a humidified incubator (5% CO_2 and 95% air), medium was changed on alternate days, and the cells split (1:3 ratio) every 5 days (0.25% trypsin, 0.53 mM EDTA solution) or when reaching 80% confluence. Primary neurons were obtained from embryonic day 18 Sprague-Dawley rats and dissociated by mild trypsination and equal numbers of cells were seeded onto 96-well plates in plating media (DMEM-12 media containing 2% B27 supplement (Invitrogen), 10% HI-FBS, 0.5 mM L-glutamine, and 25 μM L-glutamate) at a density of approximately 6×10^4 cells/well. From day 3 in vitro (DIV), cultures were maintained in feeding media (Neurobasal medium containing 2% B-27 supplement (Invitrogen) and 0.5 mM L-glutamine) in a 5% CO_2/21% O_2 atmosphere at 37°C.

RT-PCR—Total RNA was extracted from primary neurons at DIV 10 as well as from SH-SY5Y cells by utilizing Trizol reagent (Invitrogen). For primary neurons, cells from ten wells were pooled and used for RNA extraction, whose quality and quantity were assessed by spectrophotometer at 260 and 280 nM λ. Prior to RT-PCR, 1 μg of RNA was initially treated with DNase I (Ambion Inc., Austin, TX) to degrade genomic DNA. Thereafter, 50 ng of treated RNA was used for each one-step RT-PCR reaction (QIAGEN OneStep RT-PCR Kit, Valencia, CA). Specific primers for the primary neurons were: rat GLP-1R, forward: 5′ AGTAGTGTGCTCCAAGGGCAT 3′ and reverse: 5′ AAGAAAGTGCGTACCCCACCG 3′, expected PCR product is 190 bp; rat GAPDH, forward: 5′ GACCTGCAGAGCTCCAATCAAC 3′ and reverse: 5′ CACGACCCTCAGTACCAAAGGG 3′, expected PCR product is 214 bp. RNA extracted from CHO-GLP-1R cells (CHO cells permanently transfected with rat GLP-1R) was used as a positive control. RT-PCR conditions for both GLP-1R and GAPDH were: 50γC for 30 min; 95°C for 15 min followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s; then 72°C for 10 min. Total RNA was extracted from human SH-SY-5Y cells cultured on 60 mm dishes. Either 0.5 or 1 μg total RNA was used for RT-PCR. Primers for human SH-SY5Y cells were: human GLP-1R, forward: 5′ TCAAGGTCAACGGCTTATTAG 3′ and reverse: R: 5′ TAACGTGTCCCTAGATGAACC 3′, expected PCR product is 480bp. Secondary primers (a second pair of human GLP-1R primers) were: forward 5′ TTCTGCAACCGGACC 3′ and reverse 5′ CAAGTGCTCAAGCCG 3′, expected product size is 1.1kb.

Treatment—Primary neurons and human SH-SY5Y neuroblastoma cells were initially investigated for the presence of GLP-1R mRNA by RT-PCR. Thereafter, they were stimulated with GLP-1 (10 nM) to assess GLP-1R function by quantifying cAMP levels (EIA kit; Assay Designs, Ann Arbor, MI) prior to and 15 min after GLP-1 addition. Primary neurons were

challenged with $A\beta_{1-42}$ (2 μM for 24 h) after pretreatment with Ex-4 (0, 50, 100, 200, and 500 nM for 2 h) and, thereafter, assessed for viability. SH-SY5Y cultures were exposed to low (5.5 mM, 48 h) and high (50 mM, 48 h) glucose concentrations and levels of secreted Aβ in conditioned media were quantified by ELISA (ALPCO Diagnostics) in the presence and absence of Ex-4 (100 nM). SH-SY5Y cultures were additionally exposed to mild oxidative stress (H_2O_2 50 μM, 2 h) after pretreatment with vehicle, Ex-4 (100 nM) or Ex-4 (100 nM) + antagonist Ex-9-39 (10 μM). Then, 24 h after H_2O_2, challenge, cell viability was quantified using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, Madison, WI).

Animal studies and procedures

Treatment—Thirty 3xTgAD mice (13 males and 17 females), aged between 11 and 12.5 months, were included in the study and were housed under controlled 12 h light/12 h dark cycle and temperature conditions, with food and water available ad libitum. Animal studies were undertaken on approved protocols in accord with the Animal Care and Use Committees of the University of Maryland, College Park, and the National Institute on Aging Intramural Research Program, and followed NIH guidelines. At the start of the study, mice were individually housed and assigned into 4 age- and sex-matched groups: group 1: controls (3 males and 4 females) received vehicle (sodium citrate buffer) for 5 consecutive days; group 2: mice (3 males and 5 females) received low dose STZ (2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose: a well-characterized pancreatic beta-cell toxin) for 5 consecutive days; group 3: mice (3 males and 4 females) received Ex-4 only; group 4: mice (4 males and 4 females) received both STZ and Ex-4. In STZ-treated groups, STZ was freshly prepared in 0.1 mM sodium citrate solution (pH 5.5) immediately before use; mice were fasted for 4 h each day prior to i.p. administration of STZ (0.1 ml volume) for 5 consecutive days at a dose of 50 mg STZ/kg body weight. Body weight and blood glucose levels (determined from tail blood by glucometer) were monitored once every other day during the STZ treatment week and thereafter once weekly for the rest of the study. In Ex-4 treated groups (groups 3 and 4), Ex-4 (dissolved in saline) was delivered from subcutaneously implanted ALZET Micro-osmotic pumps (Model 1004, Alzet, Cupertino, CA) at a rate of 3.5 pM/kg/min. Ex-4 treatment was initiated 3 days after the final STZ dose. In control groups (group 1 and 2), saline pumps were implanted in each animal following the same surgical procedure as the Ex-4 pumps. Every 4 weeks, pumps were replaced with freshly prepared Ex-4 or saline until the close of the 16-week study. In all animals, pumps were placed subcutaneously, posterior to the scapulae. Pump implantation and replacement were performed under anesthesia (isoflurane, Abbott Laboratories, Chicago, IL) utilizing sterile procedures.

Intraperitoneal glucose tolerance test (IPGTT)—An IPGTT was performed at both week 7 and 15. Mice were fasted overnight before the glucose tolerance test. Glucose (1.5 g/kg body weight) was administered by i.p. injection. Blood glucose levels were measured from the tail vein with a glucometer immediately prior to glucose injection (0 time) and at 10, 20, 30, 60, 90, and 120 min after glucose. Between the 10 and 20 min time points, 50-100 μl of blood was collected from the tail vein, centrifuged for plasma collection and frozen (−80°C).

Insulin ELISA—Plasma was collected at baseline, during IPGTT and at the end point of the study for determination of insulin. Insulin levels were quantified using an Ultra sensitive insulin enzyme-linked immunosorbent assay kit (Crystal Chem., Chicago, IL) utilizing mouse insulin as standards and following the manufacture's protocol; each sample was analyzed in duplicate.

Hemoglobin A1c (HbA_{1C})—HbA_{1c} was determined in freshly obtained blood samples, placed on wet ice, by automated DiaSTAT analyzer (Bio-Rad Laboratories, Hercules, CA).

Tissue preparation and analyses—All animals were euthanized 16 weeks following initiation of treatment (Fig. 4A). Blood and plasma were collected, and the brain was removed and hemi-sected along the mid-line. The right hippocampus and parietal cerebral cortex were excised and immediately frozen to −80°C for analyses of AβPP, Aβ, and tau. The left hemi-sected brains were rinsed with 1x PBS and immersion fixed in 4% paraformaldehyde for 48 h. Following fixation, brains were cryoprotected in 30% sucrose at 5°C and then frozen at -80° C for processing. Thereafter, brains were sliced on a freezing microtome at a thickness of 50 μm. Serial sections were collected throughout the brain and a subset was stained with the following antibodies: Anti-Aβ 6E10 (Covance, Emeryville, CA) and Anti-Tau HT7 (Thermo Scientific, Rockford, IL). Primary antibodies were applied to free floating sections at dilutions of 1:3000 for 6E10 and 1:1000 for HT7. Stained sections were mounted onto gelatin-subbed slides and allowed to dry for at least 3 days and then cover-slipped using Permount. Slides dried for at least 1 week and were then qualitatively assessed for amyloid and tau staining [34].

Determination of AβPP, Aβ, and tau levels in brain homogenates—The homogenates from the right hippocampus were assayed for AβPP and Aβ levels as briefly described below. For AβPP, brain tissue was homogenized in 500 μl of 5M guanidine hydrochloride in 50 mM tris HCl solution, pH 8.0 [35]. Homogenized brain tissue was completely dissolved in guanidine hydrochloride solution. This clear brain homogenate was assayed first for total protein concentration and then divided into two parts: the first (~300 μl), homogenate I, was saved to assay levels of Aβ, as described below. The second (~200 μl), homogenate II, was used to quantify levels of AβPP protein, likewise described below. To remove guanidine hydrochloride, 1000 μl of ice-cold acetone was added to 200μl of the brain homogenate for precipitating the cellular protein. The solution was kept on ice for 15 min and then centrifuged at 13,000×g. The supernatant fluid was removed by pipet and the pellet was mixed with 200 μl of Tris-HCl, pH 7.4 buffer containing EDTA, EGTA, and a protease inhibitor cocktail (Roche, Indianapolis, IN) to obtain brain homogenate II. Homogenate II samples were then subjected to protein assay, as described previously [36,37]. An equal amount of protein (5 μg) was analyzed by Western blot and probed with anti-AβPP mAb 22C11 (Chemicon/ Millipore. Danvers, MA) and anti-β-actin mAbAC-15 (Sigma-Aldrich, St. Louis, MO) antibodies. Final AβPP levels were determined after adjustment with the signal from the β-actin band, which was utilized as a housekeeping control across animals.

$Aβ_{1-40}$—Quantification of $Aβ_{1-40}$ alone was undertaken as this represents the predominant Aβ species in the brain of 3xTgAD mice [32], and provides an indicator of AβPP pathway processing to Aβ. An equal volume of brain tissue homogenized in guanidine hydrochloride (homogenate I) was diluted 1:20 with ELISA diluent buffer and then subjected to a sandwich ELISA method to measure $Aβ_{1-40}$ (Biosource, Camarillo, CA) as per the manufacturer's protocol. Final Aβ levels were determined following normalization to total protein levels by Bradford assay, as described previously [36,37]. Levels of $Aβ_{1-42}$ were also measured independently using an IBL ELISA kit. However, levels proved to be below the detection level of the assay.

Tau—To measure total tau levels, the hippocampi homogenates in Tris-HCL buffer were run in SDS-polyacrylamide (10%) gel as described previously [36,37]. After transferring the proteins onto a PVDF membrane, they were blocked and then probed with monoclonal anti total tau antibody clone DC25, which recognizes all six isoforms of the human tau (rPeptide, Bogart, GA; USA) in 1:100,000 dilutions. The lower portion of the blot was independently probed with anti β-actin antibody (Chemicon). After probing one hour with HRP conjugated secondary goat anti mouse antibody followed by incubation with the ECL reagents, the blot

was exposed to an X-ray film to visualize bands. The band density of total tau was then assessed and adjusted with the quantified band density of respective β-actin band.

Data analyses

Results are expressed as mean ± SEM (where SEM is standard error of the difference between the means). Analysis of variance was carried out using StatView software (SAS Institute Inc., Cary, NC), with $p<0.05$ considered statistically significant, as assessed by Student's t-test with corrections for multiple comparisons to a single group (Dunnett's t-test) and between multiple groups (Bonferroni and Tukey's tests).

RESULTS

Human SH-SY5Y cells and rat primary neurons express a functional GLP-1R

To establish the presence of the GLP-1R, cultures were probed for the presence of GLP-1R mRNA by RT-PCR. Both cell types expressed GLP-1R mRNA (Fig. 1A, 2A). Incubation of the cells with the natural agonist, GLP-1 (10 nM), led to a significant elevation (>10-fold) of intracellular cAMP levels, assessed at 15 min (Fig. 1B, 2B).

GLP-1R stimulation reduces the vulnerability of cultured SH-SY5Y and primary neurons to Aβ/oxidative stress-induced death

Primary neurons and SH-SY5Y cells are vulnerable to oxidative stress, induced by either Aβ or H_2O_2, which results in a loss of viability (Fig. 1C, 2C). Incubation with the GLP-1R agonist, Ex-4, afforded concentration-dependent protection against Aβ in primary neurons, reaching significance at 100 nM and a maximal effect at 200 nM Ex-4. Mild oxidative stress induced by H_2O_2 provoked a small but significant loss of cell viability in SH-SY5Y cells that was fully ameliorated by Ex-4 (100 nM). This cell protection was blocked by co-administration of the GLP-1R antagonist Ex9-39 (Fig. 2C), indicating that Ex-4 generated protection was mediated via the GLP-1R.

GLP-1R stimulation reduces Aβ levels in SH-SY5Y cells in the presence of both low and high glucose levels

Human SH-SY5Y neuroblastoma cells generate and secrete Aβ in culture. To assess the actions of Ex-4 on the production of Aβ, cells were incubated in the presence and absence of Ex-4 at 5.5 mM and 50 mM concentrations of glucose, comparable to euglycemia (e.g., normoglycemia) and hyperglycemia, respectively. Ex-4 induced a decline in secreted levels of Aβ at low glucose ($p=0.06$, Student t-test) and at high glucose ($p=0.002$, Student's t-test) concentrations, with respective reductions of approximately 33% and 23% (Fig. 3).

Ex-4 ameliorates STZ-induced diabetes in 3xTg-AD mice

The study protocol is illustrated in Figure 4A. At initiation, the mean weight of mice was similar across groups, approximately 30 g, and determination of blood glucose levels demonstrated that all were euglycemic (Fig. 4B). Compared to controls, all mice administered STZ (alone or plus Ex-4 treatment) briefly lost weight during the week following STZ administration and concomitantly demonstrated a rise in blood glucose levels. Thereafter, all mice gained 1-3 g weight throughout the 16 week study and were not significantly different between groups at study end ($p>0.05$, Bonferroni t-test).

STZ induced rapid hyperglycemia in all animals. Mean blood glucose levels for STZ mice rose to and were maintained at >200 mg/dL, peaking at 261 ± 58 mg/dL (Fig. 4B). Treatment with Ex-4 (STZ+Ex-4) ameliorated this rise, and blood glucose levels were maintained between 147 to 179 mg/dL ($p<0.05$, Dunnett's t-test). Control and Ex-4 alone mice remained euglycemic

throughout the study. Two IPGTTs were undertaken, at 6 and 14 weeks following STZ administration, to characterize glucose control and insulin response following similar glucose loads. The response of animals to both IPGTTs was similar and is illustrated in Figure 5A for the 14 week test. Impaired glucose control was evident in mice administered STZ, which for STZ alone mice resulted in peak blood glucose levels of 430±48 mg/dL (Fig 4B) at 30 min and declined to 317±53 mg/dL at 120 min. Blood glucose levels were lower throughout the IPGTT in STZ animals treated with Ex-4, whose levels peaked at 347±17 mg/dL at 20 min and declined to 186±17 mg/dL at 120 min. By contrast, control and Ex-4 non-diabetic mice achieved lower peak blood glucose levels of 262±13 mg/dL and 291±33 mg/dL, respectively at 10 min, that rapidly returned to resting levels within 90 min. Assessed from the areas under the curve (AUC) (Fig. 5B), levels were not different from one another for control and Ex-4 animals ($p>0.05$, Tukey's test), were significantly elevated in the STZ group ($p<0.001$, Tukey's test) vs. control, and were partly ameliorated by Ex-4 in STZ + Ex-4 mice ($p<0.05$, Tukey's test) vs. STZ alone. On reviewing the concomitant insulin response, as illustrated in Fig. 5C, the 15-min insulin level was significantly reduced in STZ animals, compared with the control group ($p<0.05$, Tukey's test). By contrast, Ex-4 significantly reversed this in STZ animals, and levels were no different from controls ($p>0.05$, Tukey's test).

Levels of HbA_{1c} were determined at study end and, as illustrated in Figure 5D, were significantly elevated in STZ alone mice (267%) versus the control group ($p<0.001$, Bonferroni t-test). This rise was blunted in Ex-4 treated STZ mice, whose levels were significantly lower than those of STZ alone mice ($p<0.001$, Bonferroni t- test).

STZ-induced diabetes elevates brain levels of AβPP and Aβ in 3xTg-AD, and Ex-4 ameliorates this amyloidogenic effect of diabetes

Hirata-Fukae and colleagues [38] recently demonstrated that female 3xTg-AD mice, similar to those utilized in the current study, exhibit greater brain Aβ production and reduced degradation than their male counterparts. As a consequence, the brains of female 3xTg-AD mice were analyzed for AD markers in the present study. In humans, gender is an established risk factor for AD as women have been shown to have a higher prevalence and risk [39].

Illustrated in Figure 6A are cerebral cortical levels of total AβPP in mice normalized to β-actin in the same sample. Total AβPP levels were elevated (1.5-fold) in STZ female 3xTg-AD mice, compared to the control group ($p<0.05$), and this was fully reversed by Ex-4 in the STZ+Ex-4 group. Total AβPP levels in Ex-4 alone and STZ+Ex-4 mice were not significantly different from controls ($p>0.05$, Tukey's test). Soluble levels of Aβ were additionally quantified in brain (Figure 6B). Similar to levels of AβPP, soluble Aβ was elevated in STZ female mice, compared to the control group (6.8-fold, $p<0.05$, Tukey's test). This elevation was fully ameliorated in the STZ+Ex-4 group ($p<0.05$, Tukey's test), which was reduced to 84% of control mice ($p>0.05$). By comparison, the Ex-4 alone group showed a trend towards reduction and was 75% of controls ($p>0.05$).

Neither STZ-induced diabetes nor Ex-4 treatment significantly affected brain levels of total tau in female 3xTg-AD mice

Levels of total tau were assessed by Western blot analysis and, as illustrated in Figure 6C, were not significantly altered by either STZ-induced diabetes or Ex-4 treatment, relative to controls ($p>0.05$). However, a trend towards an elevation in total tau was evident in STZ-treated mice (121.7% of the control group, $p<0.05$) that was lost in the STZ+Ex-4 group (94.9% of controls, $p>0.05$).

Semi-quantitative analysis of amyloid plaques and tau immunoreactive neurons was undertaken by immunohistochemistry in the hippocampus and lateral entorhinal cortex (LEC)

of the left cerebral hemisphere, by staining with mAb 6E10 and HT7 to Aβ and tau, respectively, and is shown in Table 1. In general, in control, Ex-4 and STZ+Ex-4 mice, little to no staining of amyloid was present in either the hippocampus or lateral entorhinal cortex. When staining occurred, it was primarily within the subiculum. Two STZ alone mice, however, exhibited extensive staining in hippocampus. By contrast, minimal staining of tau was exhibited by all animals.

DISCUSSION

Mild to moderate impairments in cognitive function have been reported in both Type 1 (T1) and T2DM [40,41], and epidemiological studies have linked T2DM and hyperinsulinemia with an increased risk of developing AD (as well as vascular dementia) in elderly people [3,4,6, 9-12]. Indeed, several studies have reported that diabetic patients have a 2- to 5-fold elevated risk for AD, as compared with non-diabetic subjects [8-12]. The principal defect in T1DM is an autoimmune-mediated destruction of pancreatic β-cells, causing insulin deficiency, whereas in T2DM the prime defect is insulin resistance/desensitization, which leads to a relative insulin deficiency. In humans, T2DM often occurs in conjunction with a complex of metabolic and vascular risk factors, generally termed metabolic syndrome [41]. Separate components of this syndrome have been identified as independent predictors of cardiovascular disease, ischemic stroke, cognitive decline, and onset of dementia [42]. Multiple possible mechanisms may hence underpin the association between diabetes and AD, including direct effects of hyperglycemia to the brain, neuronal insulin receptor desenitization, and insulin-induced Aβ amyloidosis within the brain, in addition to indirect ischemic effects, such as T2DM-promoted cerebrovascular disease. In such circumstances, strategies that effectively intervene in the process(es) leading to one disease, such as T2DM, may hold promise for others, particularly when the molecular mechanisms underpinning such action share commonalities.

A strategy developed to normalize insulin signaling in T2DM involves the incretin hormone, GLP-1 that lowers blood glucose in T2DM and improves insulin receptor sensitivity [18,19] via actions on the GLP-1R. Amongst numerous deficits in AD brain, insulin receptors are likewise reported desensitized, and this condition has been termed ‘type 3 diabetes’ [43]. In brain, insulin has numerous functions, and is involved in induction of dendritic sprouting, neurogenesis, neurotransmitter regulation, and neuroprotection. Additionally, both insulin and closely related insulin-like growth factor I (IGF-I) have been reported to both regulate and be regulated by Aβ and tau [44-46], and, in preliminary studies, intranasal insulin has been reported to augment verbal memory in adults with AD and MCI who were not APOEε4 carriers [47]. Like insulin and IGF-I [48], GLP-1 and its long-acting analogue, Ex-4, enter the brain [20] and exhibit neuroprotective properties in models of ischemic stroke, PD [23,27,28], and peripheral neuropathy [49], and protect neurons from a variety of toxic insults, including kainate-induced neurotoxicity in vivo [21,25] and Fe^{2+}, Aβ, and hypoxia in cell culture [23, 26].

In this study and in accord with recent studies [21-23], we demonstrated the presence of the GLP-1R on primary cortical neurons and SH-SY5Y cells, and its functionality was confirmed by GLP-1-induced elevation of intracellular cAMP, whose pathways appear to be fundamental in neuronal survival. The pathogenic effect of high glucose is mediated to a significant degree via an increased production of reactive oxygen and nitrogen species and subsequent oxidative stress [5,10-12] that, like Aβ, can both directly inflict cellular damage and activate a number of cellular stress-sensitive pathways to induce cellular dysfunction [5-7,50]. Pretreatment with Ex-4 proved protective against Aβ- as well as oxidative stress (H_2O_2)-induced neurotoxicity (Fig. 1 and 2), representing two pathways involved in AD [5,14,15,50]. Furthermore, Ex-4 reduced the generation of Aβ in cell culture under both euglycemic and hyperglycemic conditions, suggesting that it may have utility in AD. In light of previous studies demonstrating

that GLP-1 reduced AβPP levels and was neuroprotective in neuronal cultures and acutely lowered brain Aβ levels in wild-type (wt) mice [26], our findings demonstrating long-term benefits of Ex-4 treatment for the brain in diabetic 3xTg-AD mice provide preclinical support for translational studies in human subjects with diabetes and/or AD.

To undertake this study, 3xTg-AD mice were utilized as they exhibit two critical elements of AD, amyloid plaques and NFTs [34]. Additionally, diabetes was induced in some animals by repeated low doses of the β-cell toxin, STZ, generating mice with reduced insulin levels and chronic hyperglycemia. STZ is taken up by cells via the GLUT-2 glucose transporter that is present at high levels on pancreatic β-cells but is absent at the blood-brain barrier, thereby excluding direct STZ effects on the brain following its systemic administration [51] and allowing our elucidation of the interaction of diabetes and AD pathogenesis. The STZ model has proven useful for studying the effects of chronic hyperglycemia, and its endocrinological features combine elements of T1 and T2DM [51]. In prior studies, rodent models of T1DM, involving a single i.v. administration of a large STZ dose, and of T2DM, involving db/db mice that are characterized by insulin resistance and obesity, both exhibited impaired cognitive function together with reduced synaptic plasticity, long-term potentiation (LTP), and adult neurogenesis [52-54]. Ex-4 has recently been reported to increase neurogenesis in experimental PD [27,28] and augment synaptic plasticity as well as LTP in brain slices [32], revealing multiple mechanisms by which Ex-4 can enhance brain resiliency.

Previous studies have demonstrated that the stimulation of GLP-1R signaling can ameliorate hyperglycemia, improve insulin secretion, and reduce β-cell apoptosis in db/db diabetic mice [55,18] as well as wt mice that were treated with multiple low doses of STZ [56], in a manner similar to the present study. In accord with this, chronic, steady-state administration of Ex-4 significantly reduced STZ-induced hyperglycemia in 3xTg-AD mice over the 16 week study (Fig. 4B), ameliorated the impaired glucose control evident following IPGTT (Fig. 5A,B) and lowered $\%HbA_{1C}$ (Fig. 5D). In T2DM in humans, Ex-4 has been shown to effectively translate to improved glycemic control and lowered $\%HbA_{1C}$ following acute and chronic administration [18,19]. Higher HbA_{1C} levels have been consistently associated with lower cognitive function in individuals with diabetes [57], and since $\%HbA_{1C}$ appears to be modifiable with Ex-4 therapy [18,19], it would be interesting to assess whether or not Ex-4 may impact cognition in T2DM, particularly in association with AD or mild cognitive impairment.

STZ-induced diabetes elevated the expression of soluble Aβ in the brain of female 3xTg-AD mice (Fig. 6B) and semi-quantitative immunohistochemical analysis suggested an elevation of insoluble amyloid deposits (Table 1), compared to untreated 3xTg-AD littermates, in accord with a recent report of elevated Aβ in the hippocampus and cortex of STZ treated rats [58]. AD is associated with an accumulation of parenchymal Aβ, resulting from an increase of Aβ generation and/or a decrease in its elimination from brain [13-15]. An elevation in total AβPP levels determined in the same brain samples (Fig. 6A) is suggestive of an elevated Aβ generation in the present study. However, recent studies describing reduced Aβ efflux from brain in STZ-treated rats [58] by P-glycoprotein at the blood-brain barrier, support the likelihood of a concomitant decline in clearance. Aβ elimination from brain has been reported to involve several pathways, including efflux via the low-density lipoprotein receptor-related protein 1 (LRP1) at the blood-brain barrier [59], and proteolytic enzyme degradation by neprilysin [60,61], insulin-degrading enzyme [62], endothelin-converting enzyme [61], and angiotensin converting enzyme [63]. Notably, Ex-4 fully reversed the STZ-induced Aβ and AβPP elevations evident in the brain of 3xTg-AD female mice (Fig. 6) and showed a trend toward lowering brain Aβ (25% decrease) in non-diabetic mice, in accord with the ability of acute i.c.v. GLP-1 to lower brain Aβ in wt mice [26]. The Ex-4 dose (3.5 pM/kg/min (21 μg/kg/day)) successfully utilized in our current study to ameliorate hyperglycemia and lower

Aβ in 3xTg-AD mice compares favorably with human studies focused on glycemic control (once weekly exenatide LAR: 2 mg/week (5.7 μg/kg/day for a 50 kg human) [64]. The mechanism(s) underpinning actions on AβPP and Aβ are likely multiple, as insulin and IRS-1 are key factors in many of the pathways involved in Aβ degradation [65,66], and are a focus of current studies.

A recent report by Hirata-Fukae et al. [38] indicated that the line of 3xTg-AD mice utilized in our study, which had been backcrossed onto a C57BL/6 background for 7 generations, exhibited few NFTs (Table 1), little phospho-tau in brain, and a slower progression of AD pathological features compared with the originally generated strain [33]. It was hence difficult to assess reductions in tau pathology in mice exhibiting little of such pathology (Table 1), and measurement of total tau by Western blot analysis suggested a trend toward elevation in STZ mice that was not evident after Ex-4 treatment (Fig. 6C). The amyloid hypothesis suggests that tau abnormalities are downstream of Aβ pathology [13], albeit that the relationship between amyloid and tau pathologies is not straight-forward [14,15]. A reduction of endogenous tau has been reported to ameliorate Aβ-induced neuronal dysfunction in mice [67]; conversely, Aβ reduction has been reported to ameliorate tau pathology at early, but not late, pathological stages in 3xTg-AD mice [68]. Although there is evidence that reduced insulin signaling in diabetic mice is associated with an increase in tau phosphorylation and may be rectified by insulin [69] such an increase could not be seen in the present study, and whether or not this would be amenable to regulation by GLP-1R stimulation remains to be elucidated.

Recent studies suggest that GLP-1R stimulation in brain augments synaptic plasticity and cognitive processes [31], provides neuromodulatory activity and, additionally, protects synapses from Aβ-induced dysfunction [70,32]. In light of the potent neurotrophic and neuroprotective actions that GLP-1 and Ex-4 have on neurons to increase their dendritic sprouting [24] and protect them from Aβ- and oxidative stress-induced toxicity, the cellular and *in vivo* reductions in Aβ generation characterized in the present study affirm that central GLP-1R stimulation with a long-acting agonist, such as Ex-4, may be of value in the early treatment of AD [21,71,72], and particularly when associated with T2DM or glucose intolerance.

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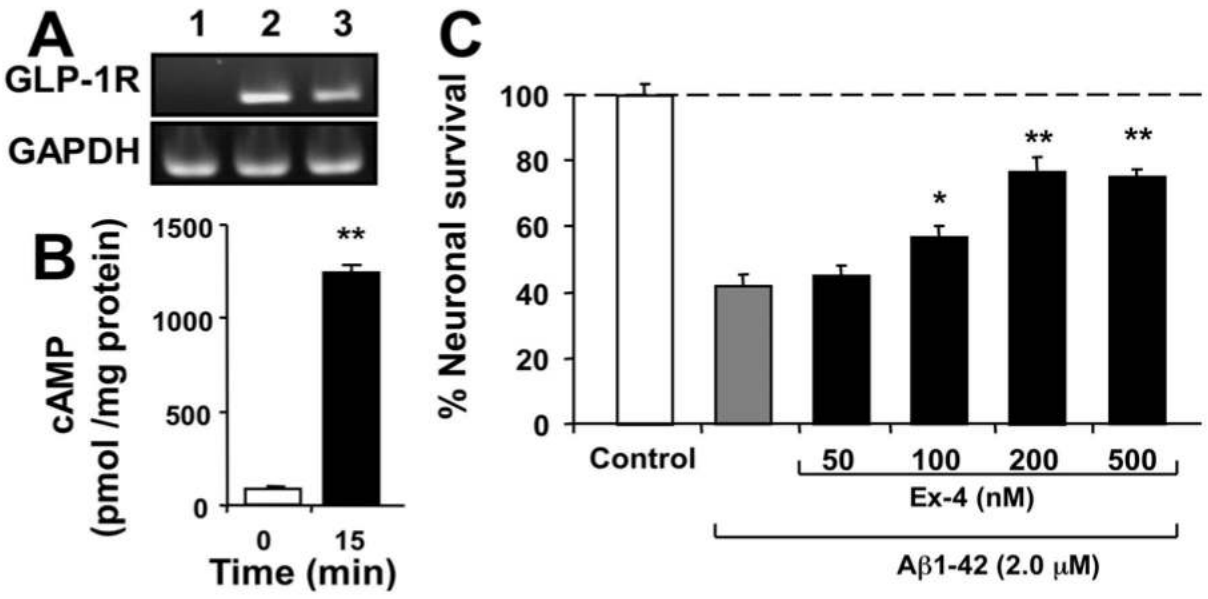

Figure 1.

The GLP-1 receptor is expressed and functional in cultured primary rat cortical neurons, and Ex-4 protects neurons against Aβ toxicity. A) One step RT-PCR showing rat GLP-1R mRNA expression in neuronal cell cultures. The expected RT-PCR product size is 190 bp. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as an external control and showed equal expression across lanes. Lane 1: negative control; Lane 2, positive control: RNA derived from CHO-GLP-1R cells (these cells were stably transfected with rat GLP-1R); Lane 3 RNA derived from primary neuron cultures. B) GLP-1 (10 nM), the natural ligand for the GLP-1R, stimulated the release of cAMP from primary neurons (N=3, **p<0.01 (Student t test) 15 min vs. zero time. C) Pretreatment with Ex-4 provided concentration-dependent protection of primary neuron cultures against a toxic insult of Aβ (2.0 μM, 2hr), N≥3 for each treatment,*p<0.05 and **p<0.01 (1-way ANOVA + post-hoc Dunnett's t-test) vs. Aβ alone.

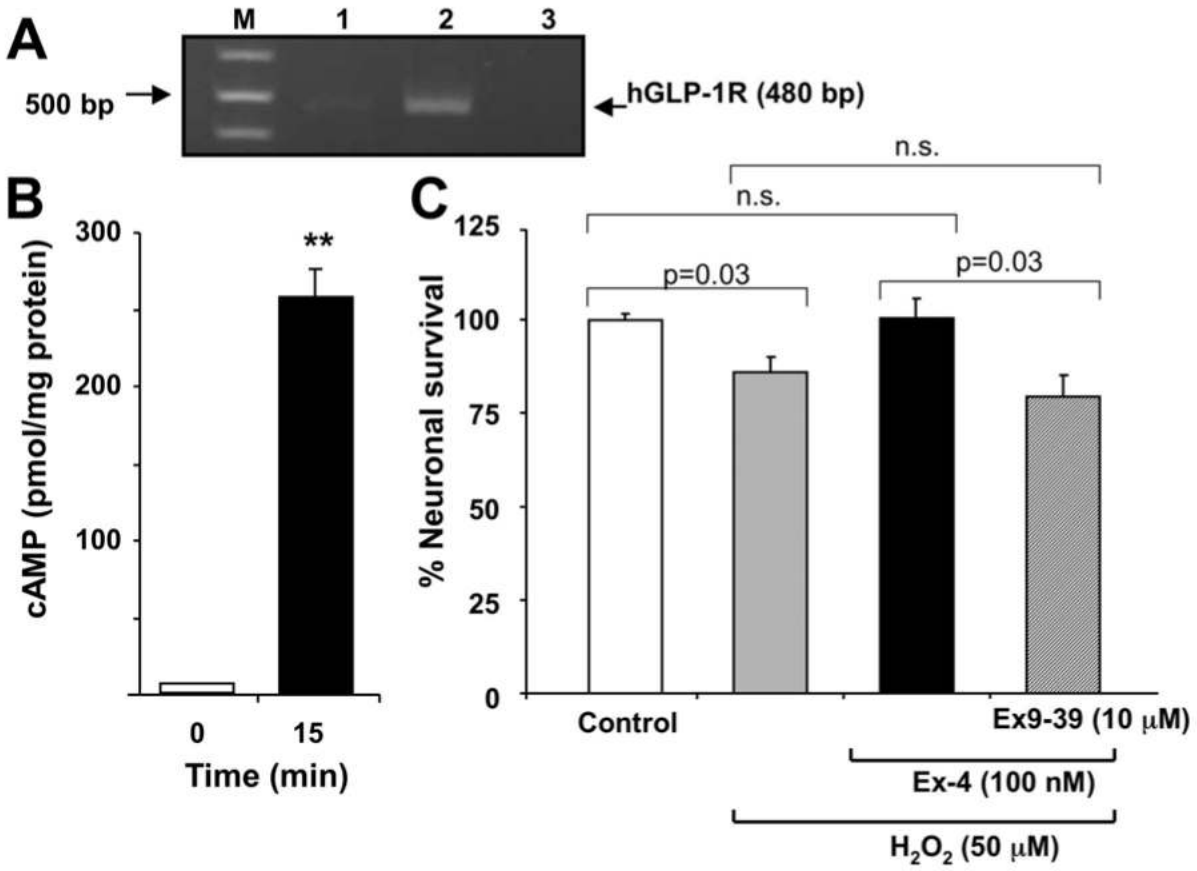

Figure 2.

Activation of GLPR-1 protects human neuroblastoma cells against oxidative injury. A) One step RT-PCR shows human GLP-1R mRNA expression in human (h) SH-SY5Y neuroblastoma cells. M: DNA marker; lanes 1 and 2: RT-PCR of hGLP-1R from 0.5 and 1 μg total RNA extracted from SH-SY5Y cells, hGLP-1R 1f/1r primers were used, which amplify a 480 bp product; lane 3: negative control. B) GLP-1 (10 nM), stimulated the release of cAMP from cultures SH-SY5Y cells (N=3, **p<0.01 (Student's t-test) 15 min vs. zero time). C) Pretreatment with Ex-4 protected SH-SY5Y cells from H_2O_2 (50≥M)-induced loss of cell viability, assessed by MTS. Compared to controls, 2 h exposure to H_2O_2 induced a 15% loss of cell viability, which was completely ameliorated by Ex-4 (100 nM). This effect was abolished by the GLP-1R antagonist, exendin-9-39 (Ex-9-39, 10 ≥M) (N≥5 for each treatment, p< 0.05 (Bonferroni t-test).

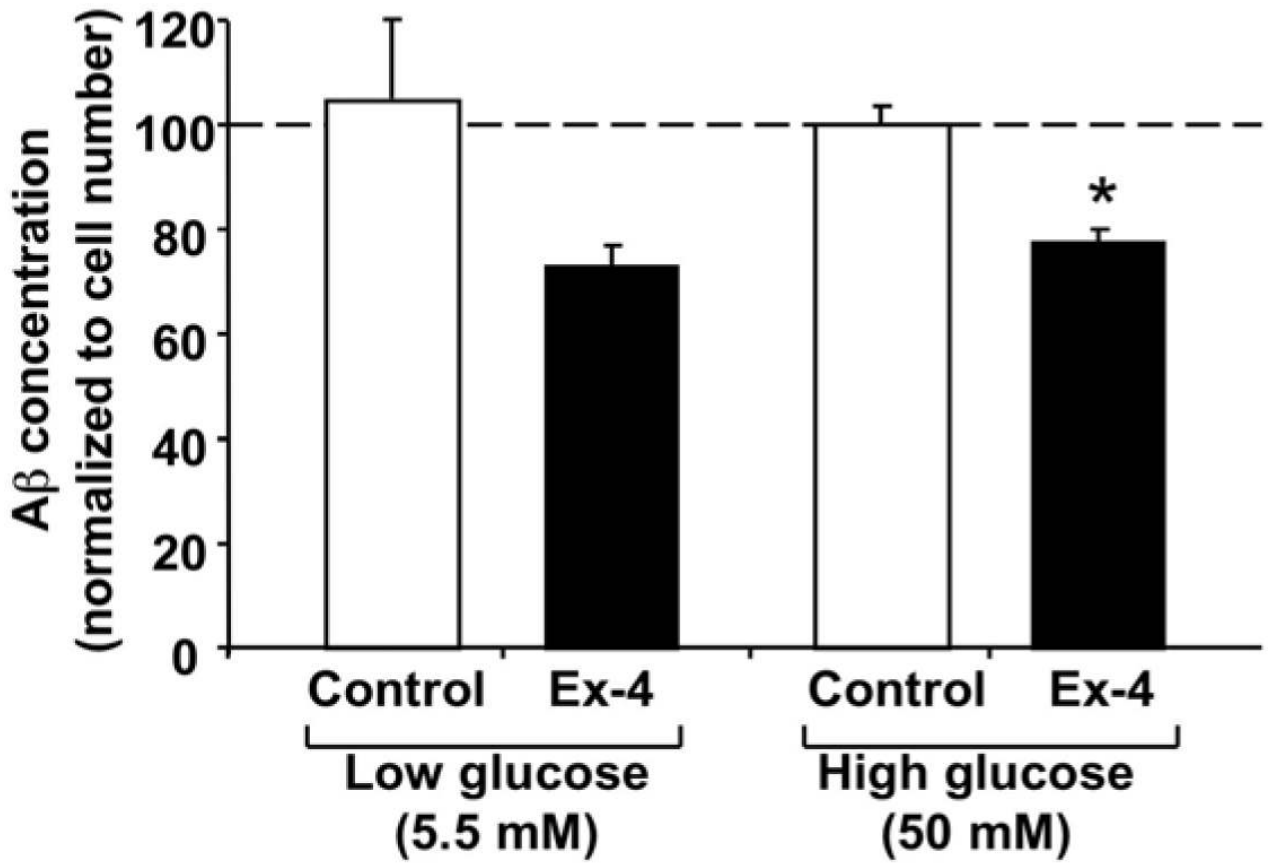

Figure 3.

Ex-4 treatment reduces the production of Aβ by cultured human neural cells. Treatment of human SH-SY5Y neuroblastoma cells with Ex-4 (100 nM) lowered their generation of Aβ, when cultured under both low (5.5 mM) and high (50 mM) glucose conditions representative of euglycemia and hyperglycemia, respectively ($N \geq 5$ for each treatment, $^*p < 0.05$ (Student t-test) vs. control).

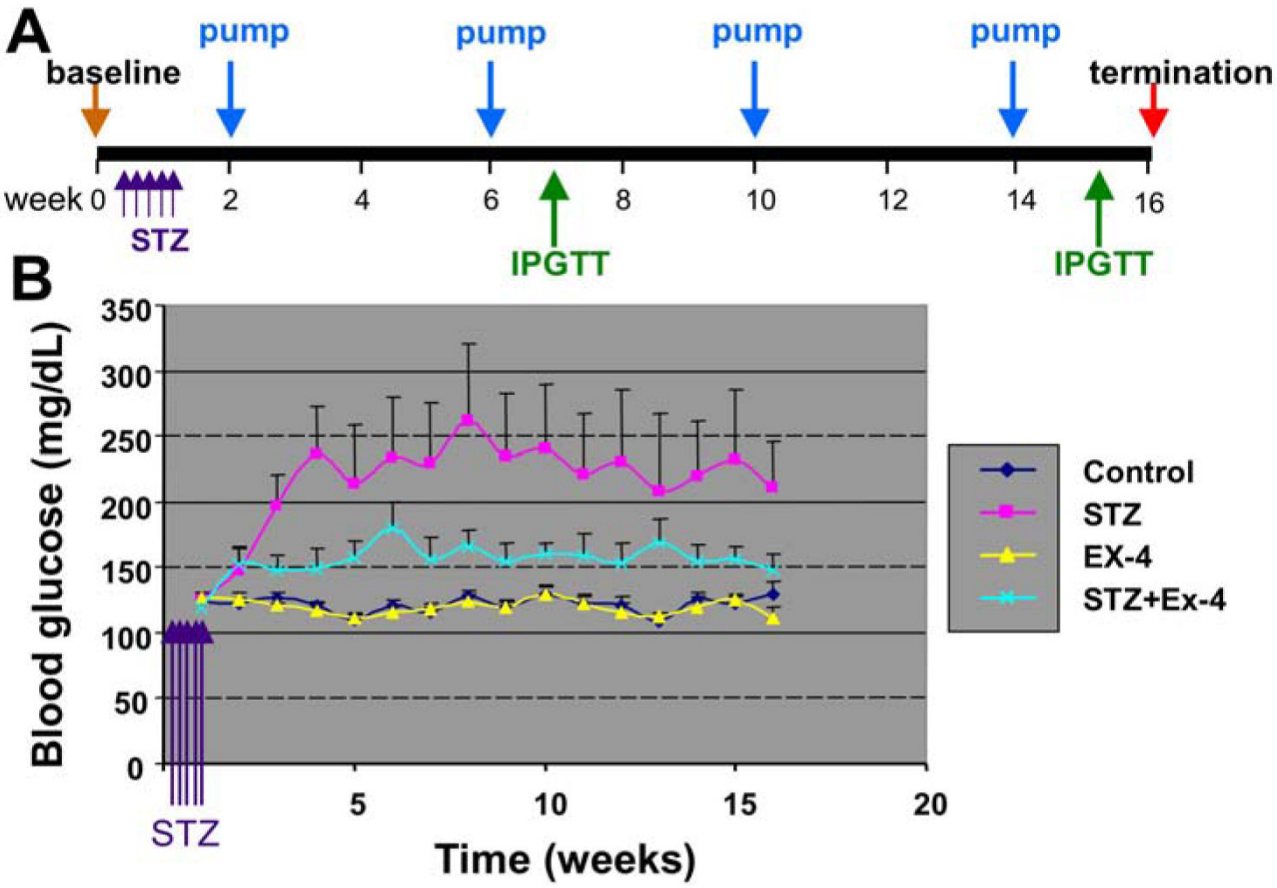

Figure 4.

Design of studies to elucidate the effects of diabetes and Ex-4 on glucose metabolism and AD-like neuropathology in 3xTg-AD mice. A) Scheme of animal study: 3xTgAD mice were assigned to four groups: (i) controls received vehicle (sodium citrate buffer) for 5 consecutive days; (ii) STZ (2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose: a well-characterized pancreatic β-cell toxin) for 5 consecutive days (50 mg/kg, i.p.); (iii) Ex-4 alone received vehicle for 5 consecutive days; (iv) STZ+Ex-4 received STZ (50 mg/kg i.p.) for 5 consecutive days. Body weight and blood glucose levels were monitored once every other day during the STZ treatment week and, thereafter, once weekly for the duration of the study (16 weeks). In Ex-4 treated groups (Ex-4 alone and STZ+Ex-4), Ex-4 was delivered by subcutaneously implanted pump initiated 3 days after the final STZ dose. Vehicle (saline) pumps were implanted into control and STZ alone groups, and the pumps were replaced every 4 weeks. Each animal received an intraperitoneal glucose tolerance test (IPGTT) on weeks 7 and 15, and was euthanized at 16 weeks. B) Time-dependent blood glucose levels (mg/dL ± SEM) in control, STZ (diabetic), Ex-4 and STZ+Ex-4 3xTg-AD mice. Both groups administered STZ (STZ and STZ+Ex-4) were hyperglycemic, compared to controls ($p<0.01$, Tukey's multiple comparison test); however, Ex-4 treatment (STZ+Ex-4) ameliorated the rise in blood glucose levels ($p<0.001$, vs. STZ alone, Tukey's test). Blood glucose levels in control and Ex-4 alone mice were no different throughout the study ($p>0.05$, Tukey's test).

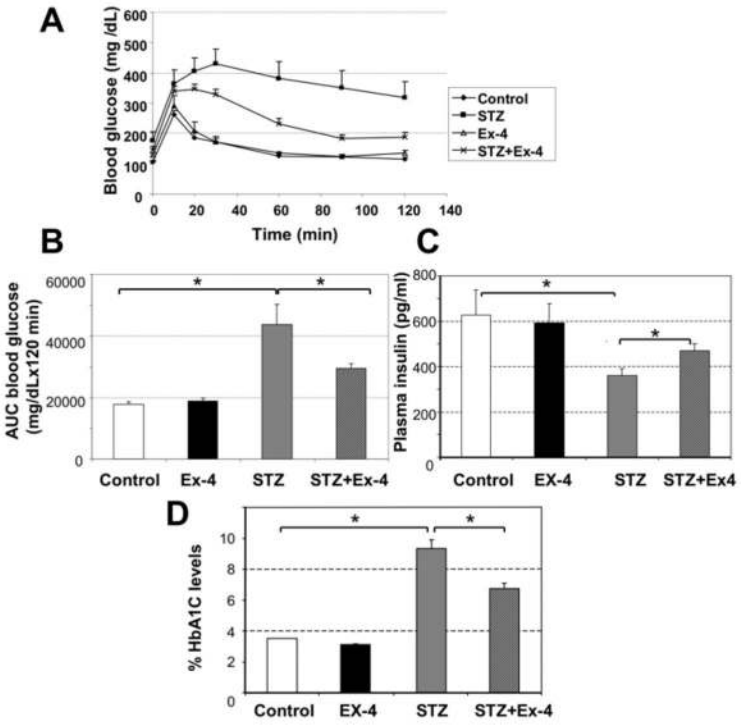

Figure 5.

Ex-4 ameliorates STZ-induced diabetes in 3xTg-AD mice. A) Time-dependent blood glucose levels (mg/dL ± SEM) following 15 week intraperitoneal glucose tolerance test (IPGTT) in control, STZ, Ex-4 and STZ+Ex-4 groups of mice. STZ animals exhibited significantly elevated levels of blood glucose, compared to controls ($p<0.05$, Tukey's multiple comparison test). These levels were significantly reduced in the STZ+Ex-4 vs. the STZ group ($p<0.05$, Tukey's test), but were not different from controls ($p>0.05$, Tukey's test). B) Area under the time-dependent blood glucose curve and (C) 15 min insulin response following week 15 IPGTT, and (D) $\%Hb_{A1C}$ at study closure (week 16). In (B), (C), and (D) STZ values were significantly different from the control group, and were ameliorated by Ex-4 (STZ+Ex-4) (*$p<0.05$, Tukey's test). The Ex-4 alone group values were no different from the control ($p>0.05$, Tukey's test).

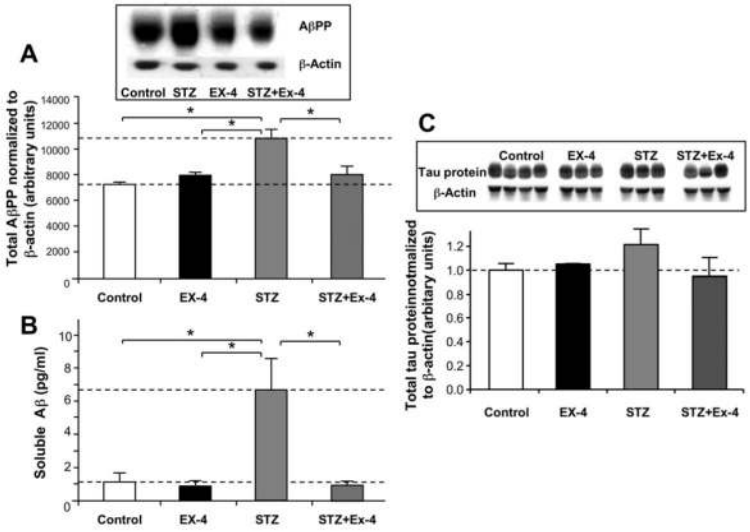

Figure 6.

Ex-4 ameliorates STZ-induced elevation in brain levels of total AβPP as well as Aβ in female 3xTg-AD mice. In female 3xTg-AD mice: A) Top: Representative Western blots of brain total AβPP (mAb 22c11) and β-actin within the same brain sample. Bottom: brain AβPP levels were normalized to β-actin and plotted for each group. STZ diabetic mice exhibited a significantly elevated level of total AβPP compared to the control group (*p<0.05, Tukey's multiple comparison test), which was reversed by Ex-4 (STZ+Ex-4) (*p<0.05 vs. STZ, Tukey's test). B) Brain Aβ levels were significantly elevated in STZ mice compared to the control group (*p<0.05, Tukey's multiple test), and were reversed by Ex-4 (STZ+Ex-4) (*p<0.05 vs. STZ, Tukey's test). C) Top: Representative Western blots of brain total tau protein (antibody clone DC25) and β-actin within the same brain sample. Levels of tau showed a trend towards elevation in STZ diabetic mice that failed to reach significance (p>0.05 STV vs. controls, Tukey's multiple test and non-parametric (Wilcoxon) analysis), which was not evident in STZ +Ex-4 mice.

Table 1

Semi-quantitative analysis of amyloid plaques and tau immunoreactive neurons within the hippocampus and lateral entorhinal cortex (LEC) of female 3xTg-AD mice following 16 weeks treatment

	Amyloid plaques[1]		**Tau immunoreactivity**[1]	
Treatment	Hippocampus	LEC	Hippocampus	LEC
Control	−	−	+	+
Control	−	−	+	+
Control	−	−	+	+
Control	−	−	+	+
STZ	+++	−	+	+
STZ	++++	+	+	+
STZ	+	−	+	+
Ex-4	++	−	+	+
Ex-4	−	−	+	+
Ex-4	++	+	+	+
STZ+Ex-4	−	−	+	+
STZ+Ex-4	+	−	+	+
STZ+Ex-4	+	−	+	+
STZ+Ex−4	++	+	+	+

[1] - no staining present

+ very limited diffuse staining (<10), no clustering

++ small amount of staining (<50), visible clustering

+++ intermediate amount of 6E10 positive staining (<100), with visible clusters

++++ extensive staining (>100), distinct clusters in multiple areas