Published in final edited form as:

J Alzheimers Dis. 2017 ; 55(2): 849–864. doi:10.3233/JAD-160656.

Improved Brain Insulin/IGF Signaling and Reduced Neuroinflammation with T3D-959 in an Experimental Model of Sporadic Alzheimer's Disease

Suzanne M. de la Monte^{a,b,c,d,e,f,g,*}, Ming Tong^{a,b,d}, Irio Schiano^h, and John Didsburyⁱ

^aLiver Research Center, Rhode Island Hospital and the Warren Alpert Medical School of Brown University, Providence, RI, USA

^bDivision of Gastroenterology, Rhode Island Hospital and the Warren Alpert Medical School of Brown University, Providence, RI, USA

^cDivision of Neuropathology, Rhode Island Hospital and the Warren Alpert Medical School of Brown University, Providence, RI, USA

^dDepartment of Medicine, Rhode Island Hospital and the Warren Alpert Medical School of Brown University, Providence, RI, USA

^eDepartment of Pathology, Rhode Island Hospital and the Warren Alpert Medical School of Brown University, Providence, RI, USA

^fDepartment of Neurology, Rhode Island Hospital and the Warren Alpert Medical School of Brown University, Providence, RI, USA

^gDepartment of Neurosurgery, Rhode Island Hospital and the Warren Alpert Medical School of Brown University, Providence, RI, USA

^hQuinnipiac University, Biomedical Sciences Department in Hamden, CT, USA

ⁱT3D Therapeutics, Inc., Research Triangle Park, NC, USA

Abstract

Background—Alzheimer's disease (AD) is associated with progressive impairments in brain insulin, insulin-like growth factor (IGF), and insulin receptor substrate (IRS) signaling through Akt pathways that regulate neuronal growth, survival, metabolism, and plasticity. The intracerebral streptozotocin (i.c. STZ) model replicates the full range of abnormalities in sporadic AD. T3D-959, an orally active PPAR-delta/gamma agonist remediates neurocognitive deficits and AD neuropathology in the i.c. STZ model.

*Correspondence to: Dr. Suzanne M. de la Monte, MD, MPH, Pierre Galletti Research Building, Rhode Island Hospital, 55 Claverick Street, Room 419, Providence, RI 02903, USA. Tel.: +1 401 444 7364; Fax: +1 401 444 2939; Suzanne_DeLaMonte_MD@Brown.edu.

Authors' disclosures available online (<http://j-alz.com/manuscript-disclosures/16-0656r1>).

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <http://dx.doi.org/10.3233/JAD-160656>.

Objective—This study characterizes the effects of T3D-959 on AD biomarkers, insulin/IGF/IRS signaling through Akt pathways, and neuroinflammation in an i.c. STZ model.

Methods—Long Evans rats were treated with i.c. STZ or saline, followed by daily oral doses of T3D-959 (1 mg/kg) or saline initiated 1 day (T3D-959-E) or 7 days (T3D-959-L) later through Experimental Day 28. Protein and phospho-protein expression and pro-inflammatory cytokine activation were measured in temporal lobe homogenates by duplex or multiplex bead-based ELISAs.

Results—i.c. STZ treatments caused neurodegeneration with increased pTau, A β PP, A β ₄₂, ubiquitin, and SNAP-25, and reduced levels of synaptophysin, IGF-1 receptor (R), IRS-1, Akt, p70S6K, mTOR, and S⁹-GSK-3 β . i.c. STZ also broadly increased neuroinflammation. T3D-959 abrogated or reduced most of the AD neuropathological and biomarker abnormalities, increased/normalized IGF-1R, IRS-1, Akt, p70S6K, and S⁹-GSK-3 β , and decreased expression of multiple pro-inflammatory cytokines. T3D-959-E or -L effectively restored insulin/IGF signaling, whereas T3D-959-L more broadly resolved neuroinflammation.

Conclusion—AD remediating effects of T3D-959 are potentially due to enhanced expression of key insulin/IGF signaling proteins and inhibition of GSK-3 β and neuroinflammation. These effects lead to reduced neurodegeneration, cognitive impairment, and AD biomarker levels in the brain.

Keywords

Alzheimer's disease; cytokines; insulin resistance; neurodegeneration; PPAR delta; rat model; T3D-959

INTRODUCTION

The sharply increased prevalence of Alzheimer's disease (AD) over the past several decades, even after correcting for age, suggests that exposure-related factors contribute to its pathogenesis [1]. Growing evidence supports the concept that AD is largely a brain metabolic disorder with molecular and biochemical features shared with diabetes mellitus [2–5]. Therefore, one potential approach to disease remediation is to utilize drugs that restore metabolic function in the brain. To accomplish this, an effective therapy should penetrate the blood-brain barrier with high efficiency, target appropriate cell types, and be effective by oral administration. T3D-959 is a small molecule, orally active, brain-penetrating PPAR- δ/γ dual nuclear receptor agonist targeted to improve neurometabolic dysfunction in AD [6,7], an aspect of the disease that is now recognized as a potential upstream driver of AD pathologies. T3D-959 was originally designed to improve insulin sensitivity and dyslipidemia in type 2 diabetes. This drug candidate has been re-positioned as a potential AD-modifying therapeutic [6,7].

Recently, T3D-959 was demonstrated to preserve spatial learning and memory and significantly reduce molecular indices of AD neurodegeneration [8], improve motor function, and reverse histopathological changes characteristic of AD [9] using an established experimental model of sporadic AD in which rats were administered intracerebral streptozotocin (i.c. STZ). PPAR δ and γ agonist isoforms are potent insulin sensitizers with

anti-inflammatory activity [10–13]. Unlike other regions of the body, the brain is almost totally dependent on glucose as an energy source and deficits in insulin signaling can negatively impact cell growth and survival, energy production, acetylcholine biosynthesis, and oxidative stress, resulting in apoptosis and impaired neuronal plasticity. The present work potentially links the initial studies demonstrating disease remediation [8, 9] with the effects of T3D-959 on insulin and IGF-1 signaling networks and pro-inflammatory cytokine activation in the temporal lobes of i.c. STZ treated rats.

METHODS

Experimental model

A sporadic AD model was generated in 4-week old male Long Evans rats (8–12/group) by intracerebral (i.c.) administration of streptozotocin (STZ) under ketamine/xylocaine anesthesia [14,15]. Control rats were given i.c. saline. Rats in each group were treated by oral gavage with 1 mg/kg/day of T3D-959 or saline [8] beginning 1 day (early treatment) or 7 days (delayed treatment) after the i.c. STZ, and continuing through Day 28 of the experiment. Upon sacrifice, temporal lobes were snap frozen and stored at -80°C for molecular and biochemical studies. In addition, in a subset of the cases, the brains were hemi-sectioned in the mid-sagittal plane; half was immersion fixed in 10% neutral buffered formalin, while the other half was micro-dissected to selectively freeze the temporal lobe. A post-fixation 3 mm-thick coronal slice that symmetrically flanked the infundibulum was embedded in paraffin. Histological sections (8 μm -thick) were stained with luxol fast blue (LFB; for myelin) plus hematoxylin and eosin (LHE).

Duplex enzyme-linked immunosorbent assays (EUSAs)

Duplex ELISAs were performed to measure immunoreactivity to Tau, phospho-Tau (S396+T205), ubiquitin, amyloid- β protein precursor (A β PP), amyloid- β fragment of A β PP (A β ₄₂), synaptophysin, and synaptosome associated protein 25 kDa (SNAP-25), and normalize the levels to the housekeeping protein, large acidic ribosomal protein (RPLPO) [16]. The approach was to first perform direct-binding ELISAs in which the proteins of interest were detected with horseradish peroxidase-conjugated secondary antibody and the Amplex UltraRed fluorophore (Invitrogen, Carlsbad, CA). After measuring fluorescence intensity, the samples were thoroughly rinsed in buffer, and then incubated with biotinylated anti-RPLPO (Proteintech Group Inc., Chicago, IL), followed by streptavidin-conjugated alkaline phosphatase with 4-Methylumbelliferyl phosphate (4-MUP) as the detection fluorophore. Fluorescence intensities (Amplex Red: Ex565/Em595; 4-MUP: Ex360/Em 450) were measured in a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). The calculated target protein/RPLPO ratios were used for inter-group statistical comparisons

Insulin/IGF-1 signaling networks

Bead-based multiplex enzyme-linked immunosorbent assays (ELISAs) were used to measure immunoreactivity to the insulin receptor (InsulinR), IGF-1 receptor (IGF-1R), IRS-1, Akt, proline-rich Akt substrate of 40 kDa (PRAS40), ribosomal protein S6 kinase (p70S6K), and glycogen synthase kinase 3 β (GSK-3 β), and pYpY1162/1163-InsulinR, pYpY1135/1136-IGF-1R, pS312-IRS-1, pS473-Akt, pT246-PRAS40, pTpS421/424-

p70S6K, and pS⁹-GSK-3 β , and a panel of 23 cytokines and chemokines (Supplementary Table 1) (Invitrogen, Carlsbad, CA). Single-plex bead-based ELISAs were used to measure mammalian target of rapamycin 1 (mTOR) and pS²⁴⁴⁸-mTOR. Samples (50 μ g protein) were incubated with magnetic beads coated with capture antibodies and immunoreactivity was detected with another epitope-specific primary antibody conjugated to phycoerythrin. Plates were read in a MAGPIX (Bio-Rad, Hercules, CA).

Statistics

Data depicted in graphs reflect group means $\pm$ S.E.M. Data were analyzed by repeated measures one-way analysis of variance (ANOVA) with the Tukey *post-hoc* multiple comparison test using GraphPad Prism 6 software (San Diego, CA).

Sources of reagents

Mouse monoclonal or rabbit polyclonal antibodies to tau (ab64193), phospho-Tau (S396) (ab109390), phospho-Tau (T205) (ab4841), ubiquitin (Ubi-1), A β PP (ab15272), A β ₄₂ (ab10148), SNAP-25 (ab66066), and synaptophysin (ab14692) were purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibody to RPLPO (RPL23 16086-1-AP) was obtained from Proteintech Inc (Chicago, IL). The Amplex UltraRed soluble fluorophore HRP-substrate and 4-MUP alkaline phosphatase substrate were from Invitrogen (Carlsbad, CA). T3D-959 was obtained from T3D Therapeutics, Inc. (Research Triangle Park, NC).

RESULTS

Characteristics of experimental i.c. STZ model

In earlier reports, we demonstrated that i.c. STZ model was associated with significantly increased blood glucose, impaired cerebellar motor and spatial learning and memory (Morris water maze testing) functions, and reduced brain weight with loss of neurons in the cerebellar and frontal cortices [8, 9]. Slice culture studies showed that i.c. STZ significantly increased A β ₄₂, S³⁹⁶+T²⁰⁵-pTau, and ubiquitin immunoreactivity in the frontal lobe [8]. Treatment with T3D-959 reduced or reversed most of these abnormalities, but the effect sizes varied with drug dose and timing in relation to the i.c. STZ, i.e., early (1 day after i.c. STZ) versus late (7 days after i.c. STZ) [8, 9]. The goal of this study was to gain a better understanding of how T3D-959 reverses or reduces neurodegeneration and neurocognitive dysfunction in the i.c. STZ model of sporadic AD. Herein, we focused on the temporal lobe and assessed expression of AD biomarkers, integrity of insulin/IGF signaling through Akt pathways, and neuroinflammation.

Histological effects of i.c. STZ with vehicle (control) versus T3D-959 treatments

Formalin-fixed, paraffin-embedded sections of temporal lobe demonstrated that in control brains, the hippocampal formation (Ammon's horn) and temporal cortex contained abundant intact neurons, rare neurons with cytological evidence of terminal injury, and intense Luxol fast blue (LFB) staining of white matter myelinated fibers (Figs. 1A, 1D, 1G, 2A, 2D, 2G). Treatment with i.c. STZ caused neuronal loss in all regions of the hippocampal formation, resulting in irregular thinning of CA1–CA4, conspicuously increased neuronal injury and death characterized by cell loss, nuclear condensation, pyknosis or apoptosis, atrophy of the

temporal cortex, and markedly reduced LFB staining of white matter, reflecting loss of myelin and/or myelinated fibers (Figs. 1B, 1E, 1H, 2B, 2E, 2H). The effects of early and late T3D-959 treatments on i.c. STZ-mediated neurodegeneration were similar. To simplify the data presentation, results corresponding to only the delayed treatment responses are shown for comparison with i.c. STZ's effects. T3D-959 treatments begun either 1 or 7 days after i.c. STZ administration resulted in conspicuously increased cellular abundance (reduced cell loss) in the hippocampal formation, reduced neuronal injury and death, increased thickness of both the temporal cortex and white matter, and increased intensity of white matter LFB staining (Figs. 1 and 2).

AD biomarker studies

Duplex ELISAs measured immunoreactivity to tau, ^{S396+T205}-pTau, A β PP, A β ₄₂, ubiquitin, synaptophysin, and SNAP-25 with results normalized to the RPLPO housekeeping protein. One-way ANOVA tests demonstrated significant inter-group differences with respect to ^{S396+T205}-pTau, A β ₄₂, ubiquitin, and synaptophysin, and a trend effect with respect to A β PP (Table 1). Significant differences were not detected for tau or SNAP-25. Results of *post hoc* Tukey tests are shown in Fig. 3. Rats treated with i.c. STZ+vehicle (Veh) had significantly increased mean temporal lobe levels of ^{S396+T205}-pTau (Fig. 3B), A β PP (Fig. 3C), A β ₄₂ (Fig. 3D), ubiquitin (Fig. 3E), and SNAP-25 (Fig. 3G), and reduced levels of synaptophysin (Fig. 3F) relative to control. In contrast, i.c. STZ+Veh had no significant effect on tau protein expression (Fig. 3A). Early treatment with T3D-959 (i.c. STZ+T3D-E) increased synaptophysin (Fig. 3F) and decreased SNAP-25 (Fig. 3G), rendering the differences from control not statistically significant. In addition, i.c. STZ+T3D-E lowered the mean level of ^{S396+T205}-pTau relative to i.c. STZ+vehicle, although the difference from control remained statistically significant (Fig. 3B). In contrast, the mean levels of A β PP, A β ₄₂, and ubiquitin were similarly elevated in the i.c. STZ+Veh and i.c. STZ+T3D-E groups. Delayed treatment with T3D-959 (i.c. STZ+T3D-L) normalized or partially abrogated i.c. STZ+Veh's effects on the mean levels of ^{S396+T205}-pTau, A β PP, A β ₄₂, synaptophysin, and SNAP-25. Although the mean levels of ubiquitin and A β ₄₂ were still elevated relative to control, they were lower than in the i.c. STZ+Veh and i.c. STZ+T3D-E groups.

Upstream signaling through the insulin and IGF-1 receptors and IRS-1

Significant inter-group differences were observed with respect to IGF-1R ($F = 4.02$, $p = 0.02$) and ^{PYPY1162/1163}-Insulin R ($F = 3.02$, $p < 0.05$), and trend effects ($0.05 < p < 0.10$) occurred with respect to IRS-1 and ^{PYPY1135/1136}-IGF-1R (Table 2). *Post-hoc* tests demonstrated significant i.c. STZ-induced reductions in IGF-1R (Fig. 4B) and a trend reduction in IRS-1 (Fig. 4C) proteins. The mean levels of insulin receptor expression were modestly also reduced by i.c. STZ, but the difference did not reach statistical significance (Fig. 4A). Insulin receptor expression was not significantly modulated by T3D-959 (Fig. 4A). Both early and delayed T3D-959 interventions tended to reduce the inhibitory effects of i.c. STZ on IGF-1 receptor expression, the differences from control remained significant (Fig. 4B). In contrast, i.c. STZ inhibition of IRS-1 protein expression was abrogated by both early and delayed administration of T3D-959 (Fig. 4C).

The i.c. STZ treatments showed an inhibitory trend on pYpY1162/1163-InsulinR expression and significantly inhibited expression of pYpY1135/1136-IGF-1R (Fig. 5B), whereas no significant or trend effects occurred with respect to S312-IRS-1 (Fig. 5C). The T3D-959 treatments were associated with further minor reductions in pYpY1162/1163-InsulinR, rendering the differences from control but not i.c. STZ+Vehicle statistically significant (Fig. 5A). In contrast, the T3D-959 treatments increased expression of pYpY1135/1136-IGF-1R levels that did not differ significantly from control (Fig. 5B). It is also noteworthy that early treatment with T3D-959 had a trend effect in elevating the mean level of pYpY1135/1136-IGF-1R above that in the i.c. STZ+Vehicle group. T3D-959 had no significant or trend effect on S312-IRS-1 (Fig. 5C). In addition, the relative levels of insulinR, IGF-1R, or IRS-1 phosphorylation were not significantly altered relative to control by either i.c. STZ or T3D-959 (Fig. 5A, 5C).

Downstream signaling through the Akt pathway

Significant inter-group differences were observed with respect to Akt, p70S6K, mTOR, and relative phosphorylated levels of pS9-GSK-3 β , pT246-PRAS40, pTpS421/424-p70S6K, and pS2448-mTOR (Table 2). In addition, trend differences were observed with respect to pS9-GSK-3 β and pT246-PRAS40. The i.c. STZ (+vehicle) exposures significantly reduced the mean levels of Akt (Fig. 4D), p70S6K (Fig. 4G), mTOR (Fig. 4H), pS9-GSK-3 β (Fig. 5E), and pS9-GSK-3 β /GSK-3 β (Fig. 6E), and increased pT246-PRAS40/PRAS40 (Fig. 6F), pTpS421/424-p70S6K/p70S6K (Fig. 6G), and pS2448-mTOR/mTOR (Fig. 6H) relative to control. In contrast, i.c. STZ had no significant effects on the mean levels of GSK-3 β (Fig. 4E) or PRAS40 (Fig. 4F) proteins, or pT246-PRAS40 (Fig. 5F), pTpS421/424-p70S6K (Fig. 5G), and pS2448-mTOR (Fig. 5H) phosphoproteins.

Both early and delayed T3D-959 interventions normalized the mean levels of Akt in i.c. STZ treated rats, and increased Akt levels relative to i.c. STZ+Vehicle (Fig. 3D). In contrast, early but not delayed T3D-959 administration reversed i.c. STZ's inhibitory effects on p70S6K (Fig. 4G) and mTOR (Fig. 4H). Delayed treatment with T3D-959 exacerbated the inhibitory effects of i.c. STZ on PRAS40 (Fig. 4F), p70S6K (Fig. 4G), and mTOR (Fig. 4H). GSK-3 β protein expression was not significantly modulated by T3D-959 (Fig. 4E).

Although both early and delayed treatments with T3D-959 increased pS473-Akt above the mean level measured in the i.c. STZ+Vehicle group, the difference was only significant for early intervention (Fig. 5D). Early and delayed T3D-959 administration abolished the significant inhibitory effects of i.c. STZ on pS9-GSK-3 β (Fig. 5E). Early T3D-959 intervention produced a trend in reduction of pTpS421/424-p70S6K (Fig. 5G) and a trend increase in pS2448-mTOR (Fig. 5H) relative to control, and significant increase in mTOR relative to T3D-L. However, delayed treatment with T3D-959 significantly reduced pS2448-mTOR expression relative to both control and i.c. STZ+Vehicle (Fig. 5G). There were no significant modulatory effects of T3D-959 on pT246-PRAS40 relative to control or i.c. STZ +Vehicle (Fig. 5F).

The relative levels of pAkt were slightly increased by early or delayed T3D-959 relative to control and i.c. STZ+vehicle, but the differences did not reach statistical significance. T3D-959 increased the mean levels of pS9-GSK-3 β /total GSK-3 β , abrogating the inhibitory

effects of i.c. STZ and normalizing the levels relative to control (Fig. 5E). Since S9 phosphorylation of GSK-3 β is inhibitory, the findings indicate that T3D-959 reduced i.c. STZ-induced GSK-3 β activation.

Early treatment with T3D-959 further increased p^{T246}-PRAS40/total PRAS40 above the level observed in the i.c. STZ + vehicle group, and rendered the difference from control to be statistically significant (Fig. 6F). In contrast, delayed treatment with T3D-959 reduced p^{T246}-PRAS40/total PRAS40, abolishing the trend effect observed in the i.c. STZ + Vehicle group. Both early and delayed treatments with T3D-959 reduced the stimulatory effects of i.c. STZ on p^{TpS421/424}-p70S6K/total p70S6K, normalizing the levels relative to control (Fig. 6G). Finally, the i.c. STZ mediated significant increase in p^{S2448}-mTOR/total mTOR was not reversed by T3D-959 (Fig. 6H).

Cytokine studies

Among the 23 cytokines measured by multiplex ELISA, significant inter-group differences were observed for 13 (EPO, IL-10, IL-12, IL-18, IL-1 α , IL-1 β , IL-2, IL-6, IL-7, M-CSF, RANTES, TNF- α , and VEGF), and a trend effect was detected for one (G-CSF) (Table 3). *Post-hoc* analysis demonstrated that i.c. STZ significantly increased EPO, IL-18, IL-1 β , IL-2, IL-4, and TNF- α relative to control. The i.c. STZ mediated increases in EPO and IL-18 were not abolished by early or delayed treatment with T3D-959, and the increased levels of IL-2, IL-4, and TNF- α persisted with either early or delayed treatment. In contrast, i.c. STZ stimulation of IL-12 did not occur in rats treated with T3D-959 (early or delayed). T3D-959 (early and delayed) significantly stimulated IL-10, IL-7, and VEGF, whereas late treatment with T3D-959 selectively and significantly suppressed IL-1b, RANTES, M-CSF, IL-5, and G-CSF, and increased IL-1a relative to control (Table 3). Further *post hoc* analysis compared the effects of i.c. STZ + Vehicle with i.c. STZ+T3D-959. Early T3D-959 treatment reduced IL-12 and increased IL-6 and VEGF relative to vehicle, delayed treatment had broader anti-inflammatory effects, significantly increasing IL-10, IL-1 α , and IL-7 and decreasing IFN- γ , M-CSF, MCP-1, and RANTES.

DISCUSSION

This preclinical study was designed to help characterize the mechanisms by which T3D-959 remediates AD-type neurodegeneration. The investigations utilized an established experimental model of sporadic AD in which Long Evans rats were administered i.c. STZ, and 1 day or 7 days later, were treated by daily oral dosing with 1 mg/kg of T3D-959 as previously reported [8]. Control rats in the i.c. STZ and i.c. saline groups received daily oral gavage with saline. The major findings in this work which was focused on the temporal lobe, were: 1) histopathological effects of i.c. STZ on the hippocampal formation, temporal cortex and temporal white matter were reduced by T3D-959 treatment; 2) T3D-959 (mainly delayed treatments) reduced expression of proteins linked to AD neurodegeneration (S³⁹⁶+T²⁰⁵-pTau, A β PP, A β ₄₂, SNAP-25), and enhanced expression of synaptophysin, which mediates synaptic plasticity in the temporal lobe; 3) the i.c. STZ impaired signaling through the IGF-1 more than through the insulin receptor; 4) i.c. STZ caused sustained impairments in signaling through Akt pathways needed for growth, plasticity, metabolism, and cell

survival; 5) i.c. STZ cause persistent neuroinflammation with broad activation of pro-inflammatory cytokines; 6) T3D-959 interventions restored multiple aspects of insulin/IGF-1/Akt pathway signaling, mainly by increasing expression of signaling proteins and increasing S9-phosphorylation of GSK-3 β . These responses were most evident in brains of animals treated after just a 1-day delay (early administration) following i.c. STZ; and 6) T3D-959 significantly reduced neuroinflammation by inhibiting pro-inflammatory and activating anti-inflammatory mechanisms. Anti-inflammatory effects of T3D-959 were more pronounced in brains treated after a 7-day delay following i.c. STZ (delayed administration).

STZ is a well-characterized toxin that is mainly used to generate models of type 1 or type 2 diabetes mellitus [17, 18]. However, low-dose i.c. STZ administration causes cognitive impairment and AD-type neurodegeneration with deficits in brain insulin and IGF signaling and energy metabolism [15, 19, 20]. The effects of i.c. STZ mimic molecular, biochemical, metabolic, and histopathological abnormalities associated with sporadic AD, including temporal cortex, white matter [21, 22], and hippocampal neurodegeneration, increased levels of S³⁹⁶+T²⁰⁵-pTau, A β PP, A β ₄₂, ubiquitin [23, 24], SNAP-25 [25], and reduced levels of synaptophysin [26], progressive impairment of brain insulin/IGF signaling and neuroinflammation with attendant increases in oxidative stress and activation of unfolded protein response pathways [2, 3, 5, 27–34], prompting the term, ‘type 3 diabetes’ to describe AD pathologies [34].

The neurodegenerative effects of i.c. STZ described herein are similar to those reported previously [15, 35], although the severity was less due to the older age of the rats used in the present study. The neuropathological abnormalities in the hippocampal formation, including atrophy with neuronal loss and white matter degeneration correspond with the impairments in spatial learning and memory in this model [8]. Furthermore, the ongoing neuronal injury and death detected in the hippocampus and temporal cortex are reminiscent of previous findings [15, 35], and most likely mark enhanced vulnerability to oxidative stress associated with terminal anesthesia as reactive glial responses were not detected. The i.c. STZ-associated increased levels of S³⁹⁶+T²⁰⁵-pTau, A β PP, A β ₄₂, ubiquitin, and SNAP-25, and reduced levels of synaptophysin mimic the molecular pathology of AD, and help reinforce the concept that impairments in brain insulin/IGF signaling through Akt pathways, together with oxidative stress and neuroinflammatory responses mediate AD-type neurodegeneration. Furthermore, the finding that T3D-959, particularly with delayed intervention, reversed, or markedly reduced, the i.c. STZ-induced molecular pathological effects corresponds with earlier findings that the insulin sensitizing PPAR- δ and PPAR- γ agonists are neuroprotective for AD-type neurodegeneration. However, T3D-959 has several major advantages over agents used in earlier studies including its dual selectivity enabling activation of both PPAR- δ and PPAR- γ nuclear receptors, high brain penetrance, oral once-a-day administration, and favorable therapeutic index [6].

This study represents the first report in which the temporal lobes from the i.c. STZ model were analyzed using multiplex bead-based ELISAs to simultaneously assess the integrity of upstream and downstream signaling through the insulin and IGF-1 receptors, IRS-1, and Akt networks. Previous studies utilized gene expression arrays, western blots, and individual molecule ELISAs. Corresponding with previous observations, a single i.c. STZ treatment

resulted in sustained inhibition of insulin and IGF-1 receptor expression. IGF-1 receptor expression was decreased due to down-regulation of both IGF-1R and pYpY^{1135/1136}-IGF-1R, whereas insulin receptor protein expression was modestly reduced and the mean levels of pYpY^{1162/1163}-InsulinR showed statistical trends for reductions. In addition, i.c. STZ inhibition of IRS-1 protein reached a statistical trend, while ^{S312}-IRS-1 was not altered by i.c. STZ. Importantly, T3D-959 normalized expression of IRS-1 and modestly increased pYpY^{1135/1136}-IGF-1R, enabling enhancement of downstream signaling through IRS-1 (and probably IRS-2). Therefore, a major positive effect of T3D-959 was to increase IRS-1 protein. This observation corresponds with previously reported effects of PPAR agonists in which increased expression of IRS-1 and IRS-2 proteins was shown to be mediated by higher levels of the corresponding mRNA transcripts [35].

The modest T3D-959-induced increases in IGF-1R and pYpY^{1135/1136}-IGF-1R and failure to normalize insulinR and pYpY^{1162/1163}-InsulinR expression most likely reflect consequences of rapid neurotoxicity, metabolic dysfunction and degeneration of insulinR and IGF-1R bearing cells following i.c. STZ treatment. Correspondingly, preservation of the relative levels of pYpY^{1162/1163}-InsulinR/insulinR and pYpY^{1135/1136}-IGF-1R/IGF-1R could be explained by loss of cells bearing those receptors rather than selective inhibition of tyrosine phosphorylation. In comparison with earlier studies, several abnormalities pertaining to upstream signaling through insulinR, IGF-1R, and IRS-1 were more striking than observed in the current study. However, the differences could be explained by the use of considerably younger rats to generate the experimental model [15, 35]. The younger rats were more susceptible to the neurotoxic and degenerative effects of i.c. STZ based on severity of histopathological lesions and deficits in spatial learning and memory. At the same time, the more robust therapeutic responses to PPAR agonists observed in previous studies were likely due to the very early (same day) treatments after administering the i.c. STZ dosing in an almost prophylactic manner. This suggests that it may be beneficial to begin therapeutic intervention prior to significant loss of insulinR and IGF-1R responsive brain cells. Of note is the profile of T3D-959 compared to other PPAR agonists, including those used in earlier studies. T3D-959 has a long plasma half-life, allows oral once-a-day administration, has high brain penetrance across the blood-brain barrier, and has selectivity as a dual PPAR- δ/γ agonist whose nuclear receptor targets regulate energy metabolism with ubiquitous PPAR delta target expression in the brain [6, 7].

The effects of i.c. STZ on downstream signaling through the Akt pathway were striking. Corresponding with previous findings, both Akt protein and pS⁴⁷³-Akt were significantly inhibited by i.c. STZ, indicating that signaling downstream would also be adversely affected. Indeed, this was the case as the mean levels of ^{S9}-GSK-3 β and relative levels of GSK-3 β phosphorylation (^{S9}-GSK-3 β /total GSK-3 β) were significantly reduced, whereas GSK-3 β protein levels were unaffected. These findings indicate that altered signaling through GSK-3 β was mediated by decreased Ser-phosphorylation rather than decreased GSK-3 β protein expression. This effect can be readily attributed to reductions in Akt signaling, which normally inhibits GSK-3 β via S9 phosphorylation. Reduced levels of S9 phosphorylation of GSK-3 β indicate higher levels of its kinase activity, leading to impairments in cellular metabolism, growth, and neuronal plasticity.

Like Akt, i.c. STZ had significant inhibitory effects on p70S6K and mTOR protein expression, but no significant effects on pTpS421/424-p70S6K and pS2448-mTOR, indicating that related impairments in signaling were due to reduced levels of protein expression. The significantly increased mean levels of pT246-PRAS40/total PRAS40, pTpS421/424-p70S6K/total p70S6K, and pS2448-mTOR/total mTOR support the concept that phosphorylation events were less impaired than protein expression. The effects of T3D-959 varied with onset of treatment such that the 1-day delay increased and normalized expression of PRAS40 p70S6K and mTOR, while delay of treatment by 7 days did not reverse i.c. STZ-inhibition of these proteins. In contrast, the effects of early T3D-959 treatments were more subtle, producing slight or trend increases (PRAS40, mTOR) or decreases (p70S6K) in the phosphoproteins, while delayed treatment resulted in no measurable change relative to the effects of i.c. STZ (pTpS421/424-p70S6K and pS2448-mTOR) or a significant reduction in phosphoprotein expression (pTpS421/424-p70S6K) relative to control and i.c. STZ. The responses of these downstream molecules to i.c. STZ were likely consequential to inhibition of IGF-1R, IRS-1, and Akt signaling, which regulate PRAS40, p70S6K, and mTOR activation. Since these molecules regulate cell motility, cell growth, cell survival, and protein translation, i.c. STZ exposure may mediate its adverse effects on the temporal lobe impairing these functions. Correspondingly, the therapeutic effects of T3D-959 were shown to be mediated in part by restoring/normalizing expression of these proteins.

The cytokine ELISA studies demonstrated that i.c. STZ had significant pro-inflammatory effects manifested by increased levels of EPO, IL-18, IL- β , IL-2, IL-4, and TNF- α relative to control. These findings indicate that pro-inflammatory cytokine activation persists long after the i.c. STZ administration, activating several immune/inflammatory cell types including hematopoietic, natural killer, macrophages, T cells, and B cells. Consistent with known anti-inflammatory and anti-oxidative stress effects of PPAR agonists, early or delayed time courses of T3D-959 intervention reduced or blocked i.c. STZ-induced pro-inflammatory cytokine activation, as manifested by the increased levels of IL-4 and IL-10, control levels of IL-12, and relative suppression of IL-1 β , RANTES, M-CSF, IL-5, and G-CSF. It is also noteworthy that delayed treatment with T3D-959 produced broader anti-cytokine/anti-inflammatory effects than early treatment. Although IL-18 levels were not reduced by T3D-959, the normalized levels of IL-12 could have been protective against activation of IFN- γ , which was significantly suppressed by delayed treatment with T3D-959.

Finally, T3D-959 stimulation of VEGF, which promotes angiogenesis, could represent a positive vascular proliferation response needed for tissue repair and ultimately enhanced local perfusion. VEGF is induced in several CNS pathologies where it may have a neuroprotective role. VEGF is reported to protect hippocampal neurons from ischemic injury [36] and enhance survival of Schwann cells [37]. In cerebellar granule neurons, Akt was phosphorylated in response to VEGF. Other studies have shown VEGF stimulation of neurons is linked to phosphoinositol-3-kinase (PI3-K) and Akt activation and neuronal protection [38]. In light of the consistent white matter vasculopathy associated with chronic degenerative or ischemic leukoencephalopathy in AD [21, 22], one of the potential therapeutic benefits of T3D-959 could be to help restore white matter integrity by promoting angiogenesis.

Conclusions

This manuscript reports the main histopathological and corresponding AD-associated molecular abnormalities in an i.c. STZ model of neurodegeneration which was previously shown to have impairments in spatial learning and memory, brain atrophy, and increased expression of AD biomarkers in the frontal lobe. In addition, this work builds on earlier observations of the therapeutic effects of T3D-959 in remediating both the brain atrophy and deficits in learning and memory in the i.c. STZ model. In addition to reversing temporal lobe and hippocampal degeneration and most molecular indices of AD-type neurodegeneration, the studies demonstrated that T3D-959 mediates its therapeutic effects by activating Akt and inhibiting GSK-3 β mechanisms, reducing neuroinflammation, and enhancing expression of synaptophysin, a mediator of synaptic plasticity. The differential effects of early versus late therapeutic interventions are noteworthy and currently under investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by AA-011431 and AG-049510 from the National Institutes of Health.

References

1. de la Monte SM, Neusner A, Chu J, Lawton M. Epidemiological trends strongly suggest exposures as etiologic agents in the pathogenesis of sporadic Alzheimer's disease, diabetes mellitus, and non-alcoholic steatohepatitis. *J Alzheimers Dis.* 2009; 17:519–529. [PubMed: 19363256]
2. de la Monte SM. Brain insulin resistance and deficiency as therapeutic targets in Alzheimer's disease. *Curr Alzheimer Res.* 2012; 9:35–66. [PubMed: 22329651]
3. de la Monte SM. Therapeutic targets of brain insulin resistance in sporadic Alzheimer's disease. *Front Biosci (Elite Ed).* 2012; 4:1582–1605. [PubMed: 22201977]
4. de la Monte SM, Longato L, Tong M, Wands JR. Insulin resistance and neurodegeneration: Roles of obesity, type 2 diabetes mellitus and non-alcoholic steatohepatitis. *Curr Opin Investig Drugs.* 2009; 10:1049–1060.
5. Talbot K, Wang HY, Kazi H, Han LY, Bakshi KP, Stocky A, Fuino RL, Kawaguchi KR, Samoyedny AJ, Wilson RS, Arvanitakis Z, Schneider JA, Wolf BA, Bennett DA, Trojanowski JQ, Arnold SE. Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *J Clin Invest.* 2012; 122:1316–1338. [PubMed: 22476197]
6. Delmedico MK, Severynse-Stevens D, Oliver WR. DB959 is a novel, dual PPAR δ/γ agonist which controls glucose and regulates triglycerides and HDLc in animal models of T2D and dyslipidemia. 69th Annual Scientific Sessions of the American Diabetes Association. 2009; 365-OR:512512.
7. Didsbury J, de la Monte SM. T3D-959: A multi-faceted disease remedial drug candidate for the treatment of Alzheimer's disease. *Alzheimers Dement.* 2015; 11:906. [PubMed: 25956989]
8. Tong M, Deochand C, Didsbury J, de la Monte SM. T3D-959: A multi-faceted disease remedial drug candidate for the treatment of Alzheimer's disease. *J Alzheimers Dis.* 2016; 51:123–138. [PubMed: 26836193]
9. Tong M, Dominguez C, Didsbury J, de la Monte SM. Targeting Alzheimer's disease neuro-metabolic dysfunction with a small molecule nuclear receptor agonist (T3D-959) reverses disease pathologies. *J Alzheimers Dis Parkinsonism.* 2016; 6:238–244. [PubMed: 27525190]

10. Fuentes L, Roszer T, Ricote M. Inflammatory mediators and insulin resistance in obesity: Role of nuclear receptor signaling in macrophages. *Mediators Inflamm.* 2010; 2010:219583. [PubMed: 20508742]
11. Glass CK, Saijo K. Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. *Nat Rev Immunol.* 2010; 10:365–376. [PubMed: 20414208]
12. Deplanque D, Gele P, Petrault O, Six I, Furman C, Bouly M, Nion S, Dupuis B, Leys D, Fruchart JC, Cecchelli R, Staels B, Duriez P, Bordet R. Peroxisome proliferator-activated receptor- α activation as a mechanism of preventive neuroprotection induced by chronic fenofibrate treatment. *J Neurosci.* 2003; 23:6264–6271. [PubMed: 12867511]
13. Sivarajah A, Chatterjee PK, Patel NS, Todorovic Z, Hattori Y, Brown PA, Stewart KN, Mota-Filipe H, Cuzzocrea S, Thiernemann C. Agonists of peroxisome-proliferator activated receptor- γ reduce renal ischemia/reperfusion injury. *Am J Nephrol.* 2003; 23:267–276. [PubMed: 12840602]
14. Hoyer S. Is sporadic Alzheimer disease the brain type of non-insulin dependent diabetes mellitus? A challenging hypothesis. *J Neural Transm.* 1998; 105:415–422. [PubMed: 9720971]
15. Lester-Coll N, Rivera EJ, Soscia SJ, Doiron K, Wands JR, de la Monte SM. Intracerebral streptozotocin model of type 3 diabetes: Relevance to sporadic Alzheimer's disease. *J Alzheimers Dis.* 2006; 9:13–33. [PubMed: 16627931]
16. Longato L, Ripp K, Setshedi M, Dostalek M, Akhlaghi F, Branda M, Wands JR, de la Monte SM. Insulin resistance, ceramide accumulation, and endoplasmic reticulum stress in human chronic alcohol-related liver disease. *Oxid Med Cell Longev.* 2012; 2012:479348. [PubMed: 22577490]
17. Bolzan AD, Bianchi MS. Genotoxicity of streptozotocin. *Mutat Res.* 2002; 512:121–134. [PubMed: 12464347]
18. Koulmanda M, Qipo A, Chebrolu S, O'Neil J, Auchincloss H, Smith RN. The effect of low versus high dose of streptozotocin in cynomolgus monkeys (*Macaca fascicularis*). *Am J Transplant.* 2003; 3:267–272. [PubMed: 12614280]
19. Plaschke K, Hoyer S. Action of the diabetogenic drug streptozotocin on glycolytic and glycogenolytic metabolism in adult rat brain cortex and hippocampus. *Int J Dev Neurosci.* 1993; 11:477–483. [PubMed: 8237464]
20. Weinstock M, Shoham S. Rat models of dementia based on reductions in regional glucose metabolism, cerebral blood flow and cytochrome oxidase activity. *J Neural Transm.* 2004; 111:347–366. [PubMed: 14991459]
21. Brun A, Englund E. Brain changes in dementia of Alzheimer's type relevant to new imaging diagnostic methods. *Prog Neuropsychopharmacol Biol Psychiatry.* 1986; 10:297–308. [PubMed: 3492011]
22. de la Monte SM. Quantitation of cerebral atrophy in preclinical and end-stage Alzheimer's disease. *Ann Neurol.* 1989; 25:450–459. [PubMed: 2774485]
23. Atkin G, Paulson H. Ubiquitin pathways in neurodegenerative disease. *Front Mol Neurosci.* 2014; 7:63. [PubMed: 25071440]
24. Oddo S. The ubiquitin-proteasome system in Alzheimer's disease. *J Cell Mol Med.* 2008; 12:363–373. [PubMed: 18266959]
25. Brinkmalm A, Brinkmalm G, Honer WG, Frolich L, Hausner L, Minthon L, Hansson O, Wallin A, Zetterberg H, Blennow K, Ohrfelt A. SNAP-25 is a promising novel cerebrospinal fluid biomarker for synapse degeneration in Alzheimer's disease. *Mol Neurodegener.* 2014; 9:53. [PubMed: 25418885]
26. Masliah E, Terry RD, DeTeresa RM, Hansen LA. Immunohistochemical quantification of the synapse-related protein synaptophysin in Alzheimer disease. *Neurosci Lett.* 1989; 103:234–239. [PubMed: 2505201]
27. Blass JP, Gibson GE, Hoyer S. The role of the metabolic lesion in Alzheimer's disease. *J Alzheimers Dis.* 2002; 4:225–232. [PubMed: 12226541]
28. Blum-Degen D, Frolich L, Hoyer S, Riederer P. Altered regulation of brain glucose metabolism as a cause of neurodegenerative disorders? *J Neural Transm Suppl.* 1995; 46:139–147. [PubMed: 8821049]
29. Hoyer S, Muller D, Plaschke K. Desensitization of brain insulin receptor. Effect on glucose/energy and related metabolism. *J Neural Transm Suppl.* 1994; 44:259–268. [PubMed: 7897397]

30. Gasparini L, Netzer WJ, Greengard P, Xu H. Does insulin dysfunction play a role in Alzheimer's disease? *Trends Pharmacol Sci.* 2002; 23:288–293. [PubMed: 12084635]
31. Hoyer S. Oxidative metabolism deficiencies in brains of patients with Alzheimer's disease. *Acta Neurol Scand Suppl.* 1996; 165:18–24. [PubMed: 8740985]
32. Craft S. Insulin resistance and Alzheimer's disease pathogenesis: Potential mechanisms and implications for treatment. *Curr Alzheimer Res.* 2007; 4:147–152. [PubMed: 17430239]
33. Rivera EJ, Goldin A, Fulmer N, Tavares R, Wands JR, de la Monte SM. Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: Link to brain reductions in acetylcholine. *J Alzheimers Dis.* 2005; 8:247–268. [PubMed: 16340083]
34. Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease—is this type 3 diabetes? *J Alzheimers Dis.* 2005; 7:63–80. [PubMed: 15750215]
35. de la Monte SM, Tong M, Lester-Coll N, Plater M Jr, Wands JR. Therapeutic rescue of neurodegeneration in experimental type 3 diabetes: Relevance to Alzheimer's disease. *J Alzheimers Dis.* 2006; 10:89–109. [PubMed: 16988486]
36. Jin KL, Mao XO, Greenberg DA. Vascular endothelial growth factor: Direct neuroprotective effect in *in vitro* ischemia. *Proc Natl Acad Sci U S A.* 2000; 97:10242–10247. [PubMed: 10963684]
37. Sundell M, Lundborg G, Kanje M. Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. *J Neurosci.* 1999; 19:5731–5740. [PubMed: 10407014]
38. Matsuzaki H, Tamatani M, Yamaguchi A, Namikawa K, Kiyama H, Vitek MP, Mitsuda N, Tohyama M. Vascular endothelial growth factor rescues hippocampal neurons from glutamate-induced toxicity: Signal transduction cascades. *FASEB J.* 2001; 15:1218–1220. [PubMed: 11344093]

Fig. 1.

Effects of i.c. STZ on hippocampal structure and responses to T3D-959 treatment. Formalin fixed paraffin embedded, Luxol fast blue-hematoxylin and eosin stained histological sections of the dorsal hippocampus (Ammon's horn) from the level of the mid-thalamus (Thal) were compared among control (A,D,G), i.c. STZ treated (B,E,H), and i.c. STZ+T3D-959 treated (7-day delay) (C,F,I) rats. Brains were harvested 4 weeks after the i.c. STZ or vehicle (control) treatments. Control samples had abundant and relatively uniform cell populations throughout CA1, CA2, most of CA3, and the dentate gyrus (DG), densely myelinated (stained blue) white matter (wm) fibers overlying Ammon's horn, and a tight (invisible) ventricular cavity in control samples. i.c. STZ resulted in irregular thickness and cellularity throughout Ammon's horn due to cell loss, markedly reduced myelin in WM, dilation of the ventricle (hydrocephalus), and ongoing neuronal injury such as shown in the circled region of H, in which numerous neurons in CA3 exhibit dense, hyperchromatic, small nuclei. T3D-959 treatment initiated 7 days after i.c. STZ moderately restored hippocampal cellularity, architecture, and WM myelin abundance, resolved the ventriculomegaly, and reduced the degree of ongoing neuronal injury (I-circled region).

Fig. 2.

Effects of i.c. STZ on hippocampal structure and responses to T3D-959 treatment. Formalin fixed paraffin embedded, Luxol fast blue-hematoxylin and eosin stained histological sections of the cerebral cortex (ctx) and white matter (wm) above Ammon's horn (Fig. 1) were compared among control (A,D,G), i.c. STZ treated (B,E,H), and i.c. STZ+T3D-959 treated (7-day delay) (C,F,I) rats. Brains were harvested 4 weeks after the i.c. STZ or vehicle (control) treatments. Cerebral cortex of control samples had abundant cellularity and densely myelinated (stained blue) white matter (wm) fibers. D,G) Higher magnification images of the cortex (Layer V—rectangle) demonstrate abundant large neurons with clear nuclei and distinct nucleoli (asterisks) and rare cells (probably neurons) with evidence of injury manifested by dense homogeneous chromatin (G; arrow). i.c. STZ resulted in cell loss manifested by increased space between neuronal cell bodies (pink areas) and markedly reduced myelin in white matter. E,H) Higher magnification images demonstrate conspicuous ongoing neuronal injury characterized by neuronal nuclear condensation (arrows), pyknosis (shrinkage; arrows), and apoptosis with pallor and loss of detail within nuclei (circled). T3D-959 treatment initiated 7 days after i.c. STZ moderately preserved neuronal density and cytoarchitecture in some regions (F1) but not all (F2), increased WM myelin staining

intensity, and reduced the abundance of neurons with ongoing neuronal injury relative to i.c. STZ.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3.

Effects of T3D-959 on AD biomarkers. Temporal lobe protein homogenates from adult male Long Evans rats administered intracerebral (i.c.) saline (Con) or STZ. The i.c. STZ group was sub-divided for daily oral treatments with 1 mg/kg of T3D-959 from 1 day (T3D-E) or 7 days (T3D-L) after the i.c. STZ through Experimental Day 28. Control rats were treated orally with saline (Veh). Each group included 8 rats. Duplex ELISAs were used to measure immunoreactivity to Tau (A), S³⁹⁶+T²⁰⁵-pTau (B), A β PP (C), A β ₄₂ (D), ubiquitin (E), synaptophysin (F), and SNAP-25 (G). Results normalized to RPLPO measured in tandem in the same wells (see Methods). Results were analyzed by one-way ANOVA (Table 1). *Post*

hoc significance tests determined the specific inter-group differences as shown in the panels (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; $\xi 0.05 < p < 0.10$).

Fig. 4.

Insulin/IGF-1 signaling through IRS-1 and downstream Akt pathway proteins. Protein homogenates were prepared from temporal lobes of adult male Long Evans rats administered intracerebral (i.c.) saline (Control; Con) or STZ. The i.c. STZ group was subdivided and treated orally with 1 mg/kg/day of T3D-959 from 1 day (T3D-E) or 7 days (T3D-L) after the i.c. STZ through Experimental Day 28. Control rats in each group were treated orally with saline (Veh). Each group included 8 rats. Bead-based ELISAs were used to measure immunoreactivity to: insulin receptor (R) (A), IGF-1R (B), IRS-1 (C), Akt (D), GSK-3β (E), PRAS40 (F), p70S6K (G), and mTOR (H). Graphs depict mean ± S.E.M.

Inter-group comparisons were made by 1-way ANOVA. *Post-hoc* Tukey test results are depicted in the graphs (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $0.05 < p < 0.10$).

Fig. 5. Phosphorylated proteins involved in insulin/IGF-1/IRS-1 signaling through Akt pathways. Temporal lobe protein homogenates were prepared from i.c. saline control (Con) and i.c. STZ treated Long Evans rats administered daily oral doses of saline (Con and STZ+Veh) or 1 mg/kg/day of T3D-959 from 1 day (T3D-E) or 7 days (T3D-L) after the i.c. treatments, through Experimental Day 28 (N = 8/group). Immunoreactivity to: pYpY1162/1163-Insulin-R (A), pYpY1135/1136-IGF-1R (B), pS312-IRS-1 (C), pS473-AKT (D), pS9-GSK-3β (E), pT246-PRAS-40 (F), pTpS421/424-p70S6K (G), and pS2448-mTOR (H) was measured using bead-based ELISAs. Graphs depict mean ± S.E.M. Data were analyzed by 1-way ANOVA and the

post-hoc Tukey test. Significant p -values and trends are depicted in the graphs ($\xi p = 0.06$; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 6.

Relative levels of insulin/IGF-1/IRS-1/Akt pathway protein phosphorylation. Temporal lobe calculated mean $\pm$ S.E.M. ratios of pYpY1162/1163-/total Insulin-R (A), pYpY1135/1136-/total IGF-1R (B), pS312-/total, IRS-1 (C), pS473-/total AKT 9D), pS9-/total GSK-3 β (E), pT246-/total PRAS40 (F), pTpS421/424-/total p70S6K (G), and pS2448-mTOR/total mTOR (H) are depicted for i.c. saline controls treated with daily oral saline as vehicle (Con), and i.c. STZ treated rats administered daily oral saline (Veh) or T3D-959 (1 mg/kg) from 1 day (T3D-E) or 7 days (T3D-L) after i.c. STZ. Data were analyzed by 1-way ANOVA and the *post-hoc*

Tukey test. Significant p -values and trends are depicted in the graphs ($\xi p = 0.06$; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

AD biomarker ELISA results-ANOVA

Proteins	F-ratio	<i>p</i> -value
Tau	0.052	N.S.
pTau (S396+T205)	4.54	0.014
A β PP	2.54	<i>0.085</i>
A β_{42}	11.38	0.0001
Ubiquitin	4.944	0.010
Synaptophysin	3.087	0.049
SNAP-25	2.00	<i>0.144</i>

Results of one-way ANOVA tests (F-ratios and *p*-values) comparing control, i.c. STZ, i.c. STZ+T3D-959 (1 day delay), and STZ+T3D-959 (7 days delay) mean levels of immunoreactivity as assessed using duplex ELISAs (see Methods). Italicized values reflect statistical trends, i.e., $0.05 < p < 0.10$.

Table 2**Multiplex ELISA results-ANOVA**

Proteins	F-ratio	<i>p</i> -value
Insulin Receptor	1.27	N.S.
IGF-1 Receptor	4.02	0.022
IRS-1	2.23	<i>0.10</i>
Akt	6.61	0.0028
GSK-3 β	0.74	N.S.
PRAS40	1.74	N.S.
p70S6K	5.47	0.006
mTOR	8.09	0.001
Phospho-Proteins	F-ratio	<i>p</i> -value
pYpY1162/1163-Insulin R	3.02	0.05
pYpY1135/1136-IGF-1R	2.46	<i>0.09</i>
pS312-IRS-1	0.87	N.S.
pS473-Akt	1.67	N.S.
pS9-GSK-3 β	2.67	<i>0.07</i>
pT246-PRAS40	2.46	0.10
pTpS421/424-p70S6K	1.23	N.S.
pS2448-mTOR	2.10	N.S.
Phospho/Total Protein Ratios	F-ratio	<i>p</i> -value
pYpY1162/1163-Insulin R/Insulin R	0.16	N.S.
pYpY1135/1136-IGF-1R/IGF-1R	0.32	N.S.
pS312-IRS-1/IRS-1	0.22	N.S.
pS473-Akt/Akt	0.82	N.S.
pS9-GSK-3 β /GSK-3 β	3.93	0.02
pT246-PRAS40/PRAS40	3.12	0.05
pTpS421/424-p70S6K/p70S6K	4.41	0.01
pS2448-mTOR/mTOR	13.18	<0.0001

Results of one-way ANOVA tests (F-Ratios and *P*-Values) comparing control, i.e. STZ, i.e. STZ+T3D-959 (1 day delay), and STZ+T3D-959 (7 days delay) mean total protein, phospho-protein, and calculated relative levels of protein phosphorylation as assessed using multiplex total and phospho-Akt pathway reagents. mTOR and pS2448-mTOR were measured using single-plex bead-based ELISAS (see Methods). Italicized values reflect statistical trends, i.e., $0.05 < p < 0.10$.

Table 3

Effects of i.c. STZ and T3D-959 on temporal lobe cytokine networks

Cytokine	Control (1)	STZ (2)	STZ+ET3D (3)	STZ+LT3D (4)	F-ratio	p-value	Versus C	Versus STZ
EPO	233.1 ± 4.28	246 ± 3.63	256.2 ± 3.60	254.9 ± 3.70	7.77	0.001	2,3,4	1
G-CSF	1.42 ± 0.06	1.31 ± 0.07	1.36 ± 0.08	1.14 ± 0.09	2.62	0.08	4	
GM-CSF	51.27 ± 4.45	45.38 ± 2.61	46.78 ± 3.52	45.13 ± 2.20	0.74	N.S.		
GRO/KC	10.38 ± 0.65	10.31 ± 0.34	11.06 ± 0.54	10.23 ± 0.30	0.63	N.S.		
IFN- γ	21.56 ± 0.73	22.22 ± 0.86	20.79 ± 0.86	19.58 ± 0.79	1.93	N.S.		4
IL-10	239.2 ± 7.02	257.3 ± 7.6	260.8 ± 5.96	279 ± 6.48	5.77	0.005	3,4	4
IL-12	15.27 ± 0.72	17.57 ± 0.52	14.84 ± 0.52	16.75 ± 0.52	4.89	0.01	2	1,3
IL-13	7.53 ± 0.23	7.7 ± 0.52	7.84 ± 0.47	6.67 ± 0.32	1.72	N.S.		
IL-18	971.3 ± 38.09	1071 ± 19.97	1067 ± 27.12	1103 ± 18.92	4.36	0.016	2,3,4	1
IL-1 α	63.28 ± 2.90	67.63 ± 4.61	70.8 ± 4.28	77.77 ± 2.94	3.39	0.039	4	4
IL-1 β	333.2 ± 8.39	367.5 ± 15.03	325.1 ± 10.89	295.4 ± 9.95	6.86	0.002	2,4	1,3,4
IL-2	89.06 ± 2.68	97.03 ± 2.61	90.62 ± 2.52	98.68 ± 2.83	3.14	0.048	2,4	1
IL-4	17.26 ± 0.42	18.51 ± 0.35	17.95 ± 0.57	18.6 ± 0.29	2.13	N.S.	2,4	
IL-5	31.23 ± 1.39	29.85 ± 1.04	29.81 ± 1.32	27.13 ± 0.67	2.27	N.S.	4	
IL-6	94.82 ± 3.42	97.39 ± 2.24	110 ± 1.58	103.7 ± 4.29	4.90	0.01	3	3
IL-7	170.4 ± 4.09	186.7 ± 8.17	191.2 ± 5.15	209.9 ± 6.15	7.12	0.002	3,4	4
IL-17A	6.09 ± 0.44	6.17 ± 0.33	5.75 ± 0.19	5.25 ± 0.24	1.78	N.S.		
M-CSF	15.27 ± 0.31	15.92 ± 0.74	14.85 ± 0.52	12.68 ± 0.50	6.79	0.002	4	4
MCP-1	52.89 ± 1.40	56.18 ± 1.46	53.46 ± 1.50	51.88 ± 1.16	1.76	N.S.		4
MIP-3 α	11.96 ± 0.46	12.25 ± 0.48	12.7 ± 0.55	12.52 ± 0.34	0.48	N.S.		
RANTES	60.96 ± 1.40	60.6 ± 1.84	62.25 ± 1.38	54.81 ± 0.87	5.47	0.007	4	4
TNF- α	145.4 ± 6.45	170.3 ± 5.03	164.3 ± 3.98	160.7 ± 6.30	3.67	0.029	2,3	1
VEGF	8.44 ± 0.24	9.32 ± 0.35	10.38 ± 0.29	9.89 ± 0.38	6.73	0.002	3,4	3

Cytokine levels were measured in temporal lobe homogenates by multiplex bead-based ELISA. Values represent ng/100 μ g protein based on standard curves using purified specific target proteins. Intergroup comparisons were made by one-way ANOVA with the Fisher *post-hoc* tests to assess significant differences from control and all other groups.

Abbreviations are defined in Supplementary Table 1. STZ, i.c. STZ; STZ+ET3D, i.c. STZ+T3D-959 delayed by 1 day post i.c. STZ; STZ+LT3D, i.c. STZ+T3D-959 delayed by 7 days post i.c. STZ.