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ORIGINAL ARTICLE

The alpha-7 nicotinic acetylcholine receptor agonist GTS-21 engages the glucagon-like peptide-1 incretin hormone axis to lower levels of blood glucose in db/db mice

Qinghe Meng MD[1] | Oleg G. Chepurny MD, PhD[2] | Colin A. Leech PhD[2] | Napat Pruekprasert BS[1] | Megan E. Molnar BS[1] | James Jason Collier PhD[3] | Robert N. Cooney MD[1] | George G. Holz PhD[2,4]

[1]Department of Surgery, State University of New York (SUNY), Upstate Medical University, Syracuse, New York, USA

[2]Department of Medicine, State University of New York (SUNY), Upstate Medical University, Syracuse, New York, USA

[3]Laboratory of Islet Biology and Inflammation, Pennington Biomedical Research Center, Baton Rouge, Louisiana, USA

[4]Department of Pharmacology, State University of New York (SUNY), Upstate Medical University, Syracuse, New York, USA

Correspondence
Robert N. Cooney, MD, Department of Surgery, SUNY Upstate Medical University, 750 East Adams Street, Suite 8141, Syracuse, NY 13210.
Email: cooneyr@upstate.edu

George G. Holz, PhD, Department of Medicine, SUNY Upstate Medical University, 505 Irving Avenue, IHP4310, Syracuse, NY 13210.
Email: holzg@upstate.edu

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Abstract

Aim: To establish if alpha-7 nicotinic acetylcholine receptor (α7nAChR) agonist GTS-21 exerts a blood glucose-lowering action in db/db mice, and to test if this action requires coordinate α7nAChR and GLP-1 receptor (GLP-1R) stimulation by GTS-21 and endogenous GLP-1, respectively.

Materials and Methods: Blood glucose levels were measured during an oral glucose tolerance test (OGTT) using db/db mice administered intraperitoneal GTS-21. Plasma GLP-1, peptide tyrosine tyrosine 1-36 (PYY1-36), glucose-dependent insulinotropic peptide (GIP), glucagon, and insulin levels were measured by ELISA. A GLP-1R–mediated action of GTS-21 that is secondary to α7nAChR stimulation was evaluated using α7nAChR and GLP-1R knockout (KO) mice, or by co-administration of GTS-21 with the dipeptidyl peptidase-4 inhibitor, sitagliptin, or the GLP-1R antagonist, exendin (9-39). Insulin sensitivity was assessed in an insulin tolerance test.

Results: Single or multiple dose GTS-21 (0.5-8.0 mg/kg) acted in a dose-dependent manner to lower levels of blood glucose in the OGTT using 10-14 week-old male and female db/db mice. This action of GTS-21 was reproduced by the α7nAChR agonist, PNU-282987, was enhanced by sitagliptin, was counteracted by exendin (9-39), and was absent in α7nAChR and GLP-1R KO mice. Plasma GLP-1, PYY1-36, GIP, glucagon, and insulin levels increased in response to GTS-21, but insulin sensitivity, body weight, and food intake were unchanged.

Conclusions: α7nAChR agonists improve oral glucose tolerance in db/db mice. This action is contingent to coordinate α7nAChR and GLP-1R stimulation. Thus α7nAChR agonists administered in combination with sitagliptin might serve as a new treatment for type 2 diabetes.

KEYWORDS
antidiabetic drug, DPP-4 inhibitor, GIP, GLP-1, glycaemic control, neuropharmacology

1 | INTRODUCTION

Type 2 diabetes (T2D) is commonly manifest as insulin resistance with attendant hyperglycaemia caused by inadequate insulin secretion from pancreatic beta cells. Treatments that normalize blood glucose in patients with T2D include medications that inhibit hepatic gluconeogenesis (metformin), enhance insulin action (metformin, pioglitazone), and inhibit renal glucose uptake (canagliflozin).[1] Additional T2D medications target the glucoregulatory glucagon-like peptide-1 (GLP-1) incretin hormone axis. These include: (1) GLP-1 receptor (GLP-1R) agonists (e.g. semaglutide) that are synthetic GLP-1 analogues,[2] and (2) dipeptidyl peptidase-4 (DPP-4) inhibitors (e.g. sitagliptin) that slow endogenous GLP-1 inactivation.[3] In T2D, these GLP-1–based medications enhance beta cell insulin secretion while also reducing beta cell apoptosis.[4] A new class of T2D medications might include GLP-1 secretagogues that stimulate GLP-1 release from intestinal L cells. In this regard, we recently proposed that alpha-7 nicotinic acetylcholine receptor (α7nAChR) agonists might serve as potent GLP-1 secretagogues with novel blood glucose-lowering properties.[5] GTS-21 (3*E*-3-(2,4-dimethoxybenylidene)-3,4,5,6-tetrahydro-2,3′-bipyridine) (DMXB-A) is one such α7nAChR agonist.[6-8] It stimulates the α7nAChR expressed on L cells of mouse primary intestinal cultures to exert a GLP-1 secretagogue action, and it also protects L cells from glucotoxicity.[9] Interestingly, mice with a global knockout (KO) of α7nAChR gene expression exhibit impaired glucose tolerance when studied on the C57BL/6J genetic background.[10] Thus the aim of the present study was to establish whether GTS-21 exerts a blood glucose-lowering action in obese, hyperglycaemic, leptin receptor-deficient db/db mice that are a model system for T2D drug discovery.[11] The main goal was to test if glucoregulatory actions of GTS-21 result from coordinate α7nAChR and GLP-1R stimulation, as predicted for this L cell GLP-1 secretagogue.

2 | MATERIALS AND METHODS

2.1 | Chemicals

GTS-21 (HY-14564A) and PNU-282987 (HY-12560A) were from MedChemExpress (MCE, Monmouth Junction, NJ). Exendin (9-39) (E7269-0.1MG) and exendin-4 (E7144-0.1MG) were from MilliporeSigma (St. Louis, MO). Sitagliptin (RS042) was from TSZ CHEM (Hinxton, Cambridgeshire, UK).

2.2 | Animals

Heterozygous (db/+) mice (strain: BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J (000642))[12] and α7nAChR KO mice (strain: B6.129S7-*Chrna7*^{tm1Bay}/J (003232))[13] were from The Jackson Laboratory (Bar Harbor, ME). GLP-1R KO mice were a gift from Drs. Daniel J. Drucker and Laurie Baggio (Lunenfeld-Tanenbaum Research Institute, Toronto, Ontario, Canada).[14] Mice were maintained on a 12-hour light–dark cycle with regular unrestricted access to Formula Diet 5008 (Lab Diet, St. Louis, MO) and free access to water. Breeding using db/+ pairings was arranged to obtain db/db mice. This study was performed in accordance with NIH and ARRIVE guidelines for the use of laboratory animals. Procedures were approved by Upstate Medical University IACUC protocols. For GTS-21 administration, single-dose studies used 10-14 week-old mice, and multiple-dose studies used 10-11 week-old mice. For additional details, see Tables S1-S5.

2.3 | Oral glucose tolerance test

All mice were fasted overnight prior to administration of glucose (2 g/kg body weight) by oral gavage. Tail blood samples were obtained before (time 0) and at 30, 60, 90, and 120 minutes after the glucose challenge for determination of blood glucose concentrations using an AimStrip Plus Blood Glucose Meter Kit (Cat. No. 37321, Germaine Laboratories, Inc., San Antonio, TX). Treatment protocols using select test agents administered prior to the oral glucose tolerance test (OGTT) are listed in section 2.5.

2.4 | Determination of body weight and food intake

The db/db mice were administered a vehicle control saline solution or GTS-21 (4 mg/kg) twice-daily (BID) by intraperitoneal injection (IP) for 8 weeks. Changes of body weight or food intake were monitored daily and are expressed as the mean ± SEM.

2.5 | Dosing regimens for the OGTT

2.5.1 | Acute treatment with GTS-21 or PNU-282987

Single IP injections of saline, GTS-21 (4 mg/kg), or PNU-282987 (10 mg/kg) were administered to overnight-fasted mice. After a 60-minute delay, the OGTT was performed.

2.5.2 | Eight-week treatment with GTS-21

Saline or GTS-21 (4 mg/kg, BID) was administered for 8 weeks by IP injection. Mice were fasted overnight, after which the OGTT was performed.

2.5.3 | Four-week treatment with PNU-282987

Saline or PNU-282987 (10 mg/kg, BID) was administered for 4 weeks by IP injection. Mice were fasted overnight, after which the OGTT was performed.

2.5.4 | Sequential administration of sitagliptin and GTS-21

Saline or sitagliptin (10 mg/kg, BID) was administered by oral gavage for 2 days. Mice were fasted overnight. On day 3, single-dose saline or GTS-21 (4 mg/kg) was administered by IP injection. After a 60-minute delay, the OGTT was performed.

2.5.5 | Co-administration of exendin (9-39) and GTS-21

Saline, GTS-21 (4 mg/kg), exendin (9-39) (85 μg/kg), or a combination of exendin (9-39) and GTS-21, were administered by single-dose IP injections to overnight-fasted mice. After a 60-minute delay, the OGTT was performed.

2.5.6 | Acute treatment with exendin-4 or GTS-21

Single-dose IP injections of saline, GTS-21 (4 mg/kg), or exendin-4 (100 μg/kg) were administered to overnight-fasted mice. After a 60-minute delay, the OGTT was performed.

2.6 | ELISA

The ELISA kit for measurement of total GLP-1 in the forms of GLP-1 (7-37), GLP-1 (7-36)amide, and GLP-1 (9-36)amide (EIAM-GLP1) was from RayBiotech (Peachtree Corners, GA). This anti-GLP-1 polyclonal antiserum recognizes an epitope within the proglucagon sequence (Uniprot P01275) corresponding to amino acid residues 108-128. It includes a portion of the mid-region of GLP-1 and extends to the C-terminus GRG residues found in GLP-1 (7-37). ELISA kits for measurement of peptide tyrosine tyrosine 1-36 (PYY1-36; EIAM-PYY) or insulin (ELM-Insulin) were from RayBiotech. The ELISA kit (27702) for measurement of active glucose-dependent insulinotropic peptide (GIP) (1-42) was from IBL-America (Minneapolis, MN). Glucagon was detected using an ELISA kit (10-1281-01) from Mercodia (Winston-Salem, NC).

2.7 | Dosing regimens for immunoassays monitoring plasma hormone levels

2.7.1 | Sequential administration of sitagliptin and GTS-21

Saline or sitagliptin (10 mg/kg, BID) was administered by oral gavage for 2 days. Mice were then fasted overnight. On day 3, saline or GTS-21 (4 mg/kg) was administered by IP injection. After a 30-minute delay, glucose was delivered by oral gavage. Thirty minutes later, blood samples were collected.

2.7.2 | Acute treatment with GTS-21 and PNU-282987

Single IP injections of saline, GTS-21 (4 mg/kg), or PNU-282987 (10 mg/kg) were administered to overnight-fasted mice. After a 30-minute delay, glucose was delivered by oral gavage. 30 minutes later, blood samples were collected.

2.8 | Insulin tolerance test

Mice were administered saline or GTS-21 (4 mg/kg, IP, BID) for 2, 4, or 6 weeks. On the morning of each experiment, an additional IP dose of GTS-21 (4 mg/kg) was administered. Mice were then fasted for 6 hours. Next, tail blood samples were obtained at time 0 minutes for determination of fasting blood glucose levels. Insulin (1 IU/kg, IP) was then administered, and additional blood was collected for determination of glucose levels at 15, 30, 60, and 90 minutes.

2.9 | Pancreas histology and immunohistochemistry

After fixation in 10% neutral buffered formalin for 24-48 hours, pancreatic tissue isolated from 17-18 week-old db/db mice was embedded in paraffin. Five-micron sections were cut and placed onto positively charged glass slides for immunohistochemical (IHC) analysis.[15] Detection of insulin was performed using a Bond-Max Automated IHC Staining System and a Bond Polymer Refine detection kit (DS9800; Leica Biosystems Inc., Buffalo Grove, IL). The primary polyclonal antiserum was guinea pig anti-insulin (1:800 dilution, 180067, Invitrogen, Grand Island, NY). The secondary polyclonal antiserum was horseradish peroxidase-conjugated rabbit anti-guinea pig (1:800 dilution, A5545, MilliporeSigma). Stained sections were imaged at 20× magnification using a NanoZoomer digital slide scanner (Hamamatsu, Bridgewater, NJ). Visiopharm VIS software version 5.0.5. (Hørsholm, Denmark) was used to simultaneously calculate the insulin positive area and islet fraction percentage ([total islet area]/[total slice area]) × 100.

2.10 | Statistical analysis

Data for each experiment are expressed as either mean ± SEM or as box and whisker plots. In the box and whisker plots, the box represents the 25%-75% range, the line within the box is the mean value, and the whiskers are the minimum and maximum values. The sample size for each experimental group is presented in the figure legends. Student's *t*-test or one-way analysis of variance (one-way ANOVA) with Bonferroni's multiple comparisons tests were used to determine the significance of group differences. Differences among groups were considered significant at P less than .05. All data were obtained from three or more independent experiments. The area-under-the-curve (AUC) for OGTTs was calculated using the trapezoidal rule. All

statistical analyses were carried out with GraphPad Prism (V 9.0) (GraphPad Software, San Diego, CA).

3 | RESULTS

3.1 | GTS-21 fails to alter body weight and food intake but improves oral glucose tolerance

The ability of GTS-21 to alter body weight and food intake, or to improve oral glucose tolerance, was evaluated using db/db mice. Administration of GTS-21 for 8 weeks (4 mg/kg, IP, BID) failed to alter body weight and food intake in comparison with mice administered saline (Figure 1A). However, a significant glucoregulatory action of GTS-21 was noted in an OGTT using chronic or acute dosing protocols (Figure 1B). Chronic administration of GTS-21 (4 mg/kg, IP, BID, 8 weeks) to db/db mice improved glucose tolerance by lowering levels of blood glucose at the 30-, 60-, 90-, and 120-minute OGTT time points (Figure 1C). Accompanying AUC analysis illustrates this cumulative effect manifest as a 29% reduction of the AUC value measured during the 0-120 minute time interval (Figure 1D). Similarly, a single dose of GTS-21 (4 mg/kg, IP) significantly lowered blood glucose levels at the 30- and 60-minute OGTT time points (Figure 1E). This improved glucose tolerance was measured as a 33% reduction of the cumulative AUC (Figure 1F). Glucoregulatory actions of 8-week and single-dose GTS-21 were also measurable when evaluating data for male or female mice (Figure S1). Moreover, the single-dose glucoregulatory action of GTS-21 was significant in the OGTT using heterozygous db/+ mice that are lean and not hyperglycaemic (Figure 1E,F). Importantly, the glucoregulatory action of GTS-21 in db/db mice was dose-dependent. This was established when testing single-dose GTS-21 administered as 0.5, 2, 4, or 8 mg/kg in the OGTT (Figure 1G,H). Collectively, these findings are understandable if glycaemia in db/db mice is physiologically regulated by neuronal acetylcholine acting at the α7nAChR, an effect reproduced by GTS-21 in assays of oral glucose tolerance.

3.2 | PNU-282987 replicates the action of GTS-21 in the OGTT

If α7nAChR mediates the glucoregulatory action of GTS-21, it is expected that the structurally unrelated α7nAChR agonist PNU-282987 will exert a similar effect in the OGTT.[16] This was in fact the case. Administration of PNU-282987 (10 mg/kg, IP, BID) for 4 weeks to db/db mice led to a blood glucose-lowering effect (Figure 2A) that was significant when quantified by AUC analysis (Figure 2B). Furthermore, an acute improvement of glucose tolerance was measured in response to a single dose of PNU-282987 (10 mg/kg, IP) administered to db/db mice (Figure 2C), an action that was also significant (Figure 2D). Note that for these assays, the GLP-1R agonist exendin-4 (100 μg/kg) served as a positive control (Figure 2C,D).[17] Finally, single-dose administration of PNU-282987 (10 mg/kg, IP) to heterozygous db/+ mice also led to a significant blood glucose-lowering effect (Figure 2E,F). These studies with PNU-282987 complemented findings obtained with GTS-21 and provided independent confirmation for α7nAChR-mediated glucoregulatory actions of α7nAChR agonists.

3.3 | Cooperative actions of GTS-21 and sitagliptin in the OGTT

GTS-21 stimulates GLP-1 secretion from primary cultures of mouse intestinal cells enriched with enteroendocrine L cells that synthesize GLP-1.[9] If GTS-21 exerts an analogous action in vivo, then GLP-1 might mediate the action of GTS-21 to lower levels of blood glucose. If this is the case, then the blood glucose-lowering action of GTS-21 should be enhanced by sitagliptin, a DPP-4 inhibitor that slows metabolic inactivation of GLP-1.[3] To test this hypothesis, the OGTT was performed using db/db mice administered GTS-21 alone, sitagliptin alone, or GTS-21 in combination with sitagliptin. The dosing regimen was designed so that saline or sitagliptin (10 mg/kg, BID) was administered by oral gavage for 2 days, after which the mice were fasted overnight. On day 3, single-dose GTS-21 (4 mg/kg, IP) or saline was administered 60 minutes prior to oral glucose. Using this approach, it was shown that GTS-21 alone, or sitagliptin alone, exerted a blood glucose-lowering effect of comparable magnitude (Figure 3A) and significance (Figure 3B). As predicted, combined administration of GTS-21 and sitagliptin led to an enhanced glucoregulatory effect (Figure 3A) that was also significant (Figure 3B). Thus the GLP-1–mediated glucoregulatory action of GTS-21 was enhanced by sitagliptin.

3.4 | Opposing actions of GTS-21 and exendin (9-39) in the OGTT

If GTS-21 improves glucose tolerance by acting as a GLP-1 secretagogue, its glucoregulatory action in the OGTT might be counteracted by the GLP-1R antagonist exendin (9-39).[17] However, if the GLP-1 secretagogue action of GTS-21 is sufficiently strong, the resultant high concentrations of released GLP-1 may outcompete and override the antagonist action of exendin (9-39) at the GLP-1R. Both predictions are compatible with findings presented here in which db/db mice were administered a single IP dose of GTS-21 alone, exendin (9-39) alone, or GTS-21 in combination with exendin (9-39) prior to the OGTT (Figure 3C,D). Note that for these mice, GTS-21 alone (4 mg/kg) exerted a blood glucose-lowering action that was significant, whereas exendin (9-39) (85 μg/kg) instead significantly raised blood glucose levels. The action of exendin (9-39) alone to raise blood glucose levels is expected because it blocks baseline stimulatory effects of endogenous GLP-1 (or possibly glucagon; see Discussion) at the beta cell GLP-1R. When GTS-21 and exendin (9-39) were administered in combination, blood glucose levels in the OGTT returned to near normal at the 30-, 60-, 90-, and 120-minute time points

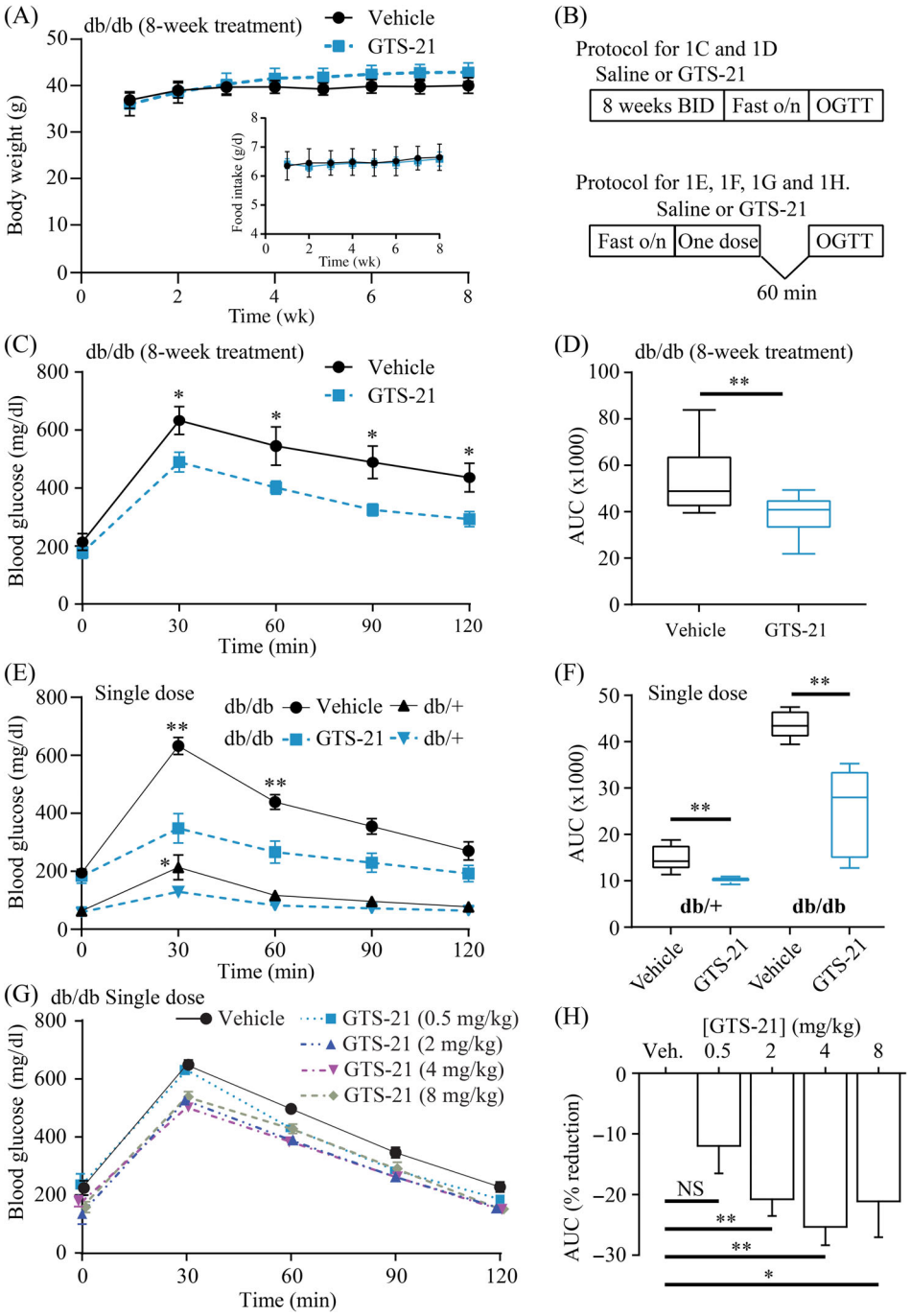

FIGURE 1 Evaluation of GTS-21 action in assays of body weight and oral glucose tolerance. A, In comparison with control mice administered saline solution (Vehicle), db/db mouse body weight and food intake were not significantly altered during an 8-week treatment with GTS-21 (4 mg/kg, IP, BID). N values for db/db mice are: 10 vehicle-treated; nine GTS-21–treated. B, Oral glucose tolerance test (OGTT) protocols used in C, E, and G. BID, twice-daily dosing; o/n, overnight. C and D, Eight-week administration of GTS-21 (4 mg/kg, IP, BID) to db/db mice improved glucose tolerance in the OGTT. Changes in tail blood glucose concentration after oral gavage with glucose (2 g/kg) are shown in C, and the corresponding area-under-the-curve (AUC) analysis is presented in D. N values for db/db mice are: 10 vehicle-treated; nine GTS-21–treated. E and F, Single-dose GTS-21 (4 mg/kg, IP) improved glucose tolerance in the OGTT measured as a blood glucose-lowering effect in db/db or db/+ mice (E) and quantified by AUC analysis (F). N values for db/db mice are: five vehicle-treated; five GTS-21–treated. N values for db/+ mice are: five vehicle-treated; five GTS-21–treated. G and H, GTS-21 (0.5-8.0 mg/kg, IP) acted in a dose-dependent manner to improve glucose tolerance in the OGTT using db/db mice (G) and quantified by AUC analysis (H). N values are: vehicle (nine); 0.5 mg/kg (three); 2 mg/kg (three); 4 mg/kg (nine); 8 mg/kg (three). For all panels, the values are mean ± SEM with accompanying box and whiskers plots. *$P < .05$ and **$P < .01$. Data in C and D, and E and F, are reanalysed in Figure S1 for male mice only, or female mice only. See Table S1 for detailed information concerning the numbers of male and female mice, mean body weights, and body weight ranges for these mice

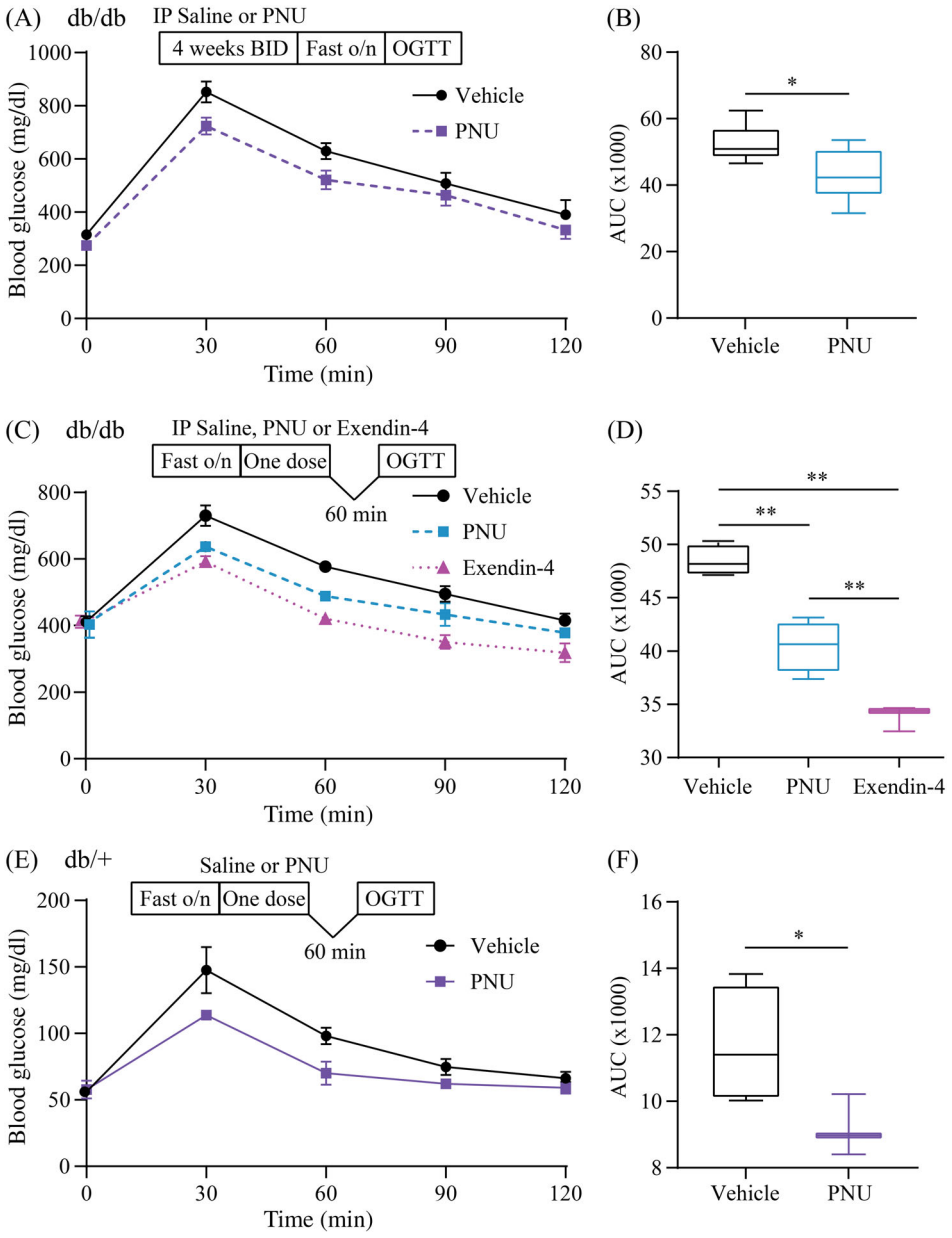

FIGURE 2 PNU-282987 replicates the action of GTS-21 in the oral glucose tolerance test (OGTT). A and B, Four-week administration of PNU-282987 (PNU, 10 mg/kg, IP, BID) exerted a significant blood glucose-lowering effect in the OGTT using db/db mice administered glucose (2 g/kg) by oral gavage (A) and quantified by AUC analysis (B). N values for db/db mice are: 10 vehicle-treated; nine PNU-282987–treated. C and D, Single-dose PNU-282987 (10 mg/kg, IP) exerted a significant blood glucose-lowering effect in db/db mice (C), as quantified by AUC analysis (D). Single-dose glucagon-like peptide-1 receptor (GLP-1R) agonist exendin-4 (100 μg/kg, IP) served as a positive control. N values for db/db mice are: four vehicle-treated, four PNU-282987–treated; and four exendin-4–treated. E and F, A significant blood glucose-lowering effect of single-dose PNU-282987 (10 mg/kg, IP) was also measured in heterozygous db/+ mice (E), as quantified by AUC analysis (F). N values for db/+ mice are: four vehicle-treated; three PNU-282987–treated. For all panels, the values are mean ± SEM with accompanying box and whiskers plots. *$P < .05$ and **$P < .01$. See Table S2 for detailed information concerning the numbers of male and female mice, mean body weights, and body weight ranges for these mice

(Figure 3C). Thus no significant change of glucose tolerance was measured (Figure 3D). Collectively, these findings point to a probable role for GLP-1 as mediator of α7nAChR glucoregulatory action. This hypothesis was tested more directly using α7nAChR and GLP-1R KO mice (see below).

3.5 | GTS-21 raises levels of glucoregulatory hormones

Potential secretagogue actions of GTS-21 administered alone, or in combination with sitagliptin, were evaluated in vivo using db/db mice. After overnight fasting, single-dose GTS-21 (4 mg/kg, IP) was administered. Next, after a 30-minute delay, glucose was delivered by oral gavage. Thirty minutes later, blood samples were collected. GTS-21 significantly raised levels of total GLP-1, active GIP, PYY1-36, glucagon, and insulin in comparison with mice administered saline (Figure 3E-I). Using this same approach, it was established that the α7nAChR agonist PNU-282987 also raised circulating levels of GLP-1 and insulin in db/+ mice (Figure S2). Single-dose sitagliptin (10 mg/kg, BID) was instead administered for 2 days by oral gavage. Mice were fasted overnight, and on day 3, single-dose GTS-21 (4 mg/kg, IP) was administered, after which the oral glucose gavage and blood draw protocols were performed. Circulating levels of GLP-1 and GIP were elevated following administration of sitagliptin (Figure 3E,F), and even higher levels of GLP-1 were measured in mice co-administered GTS-21 and sitagliptin (Figure 3E). Thus GTS-21 may act at α7nAChR on L and K cells to stimulate GLP-1 and GIP release, after which these hormones act at their cognate receptors on islet beta cells to stimulate insulin secretion.

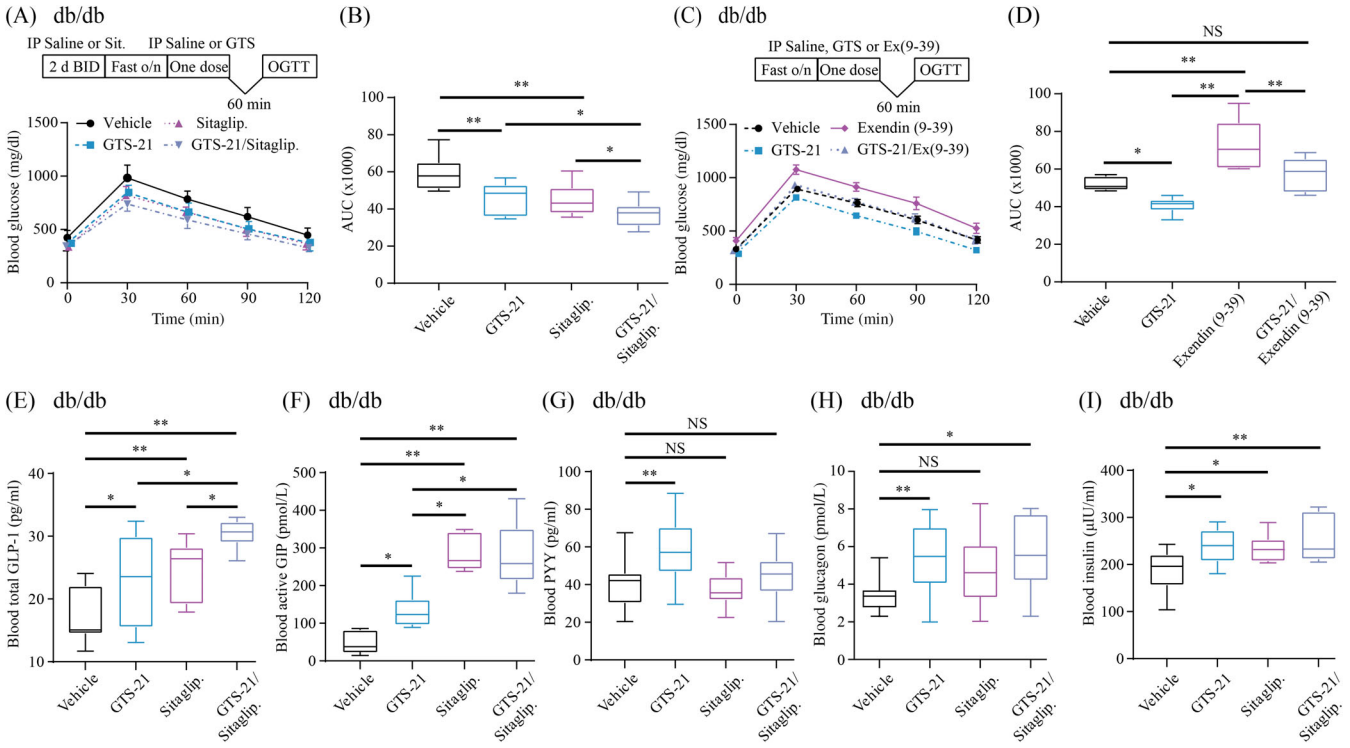

FIGURE 3 Glucoregulation in mice administered GTS-21, sitagliptin, or exendin (9-39). A and B, Sitagliptin (10 mg/kg, BID) or vehicle saline control was administered to db/db mice for 2 days by oral gavage. On day 3, overnight-fasted mice were administered GTS-21 (4 mg/kg, IP) or saline as a single dose 60 minutes prior to oral glucose (2 g/kg). GTS-21 or sitagliptin alone significantly lowered blood glucose levels in the oral glucose tolerance test (OGTT), and their combined administration had an additive effect (A), as quantified by AUC analysis (B). N values for db/db mice are: nine vehicle-treated; nine GTS-21–treated; 10 sitagliptin-treated; and 11 GTS-21/sitagliptin-treated. C and D, Single-dose exendin (9-39) (85 μg/kg, IP) or GTS-21 (4 mg/kg, IP) was administered to db/db mice alone or in combination as a single dose 60 minutes prior to oral glucose (2 g/kg). GTS-21 alone lowered blood glucose levels, whereas exendin (9-39) exerted an opposite effect in the OGTT. Note that blood glucose levels in the OGTT matched the vehicle control (saline, IP) values at all time points after combined administration of GTS-21 and exendin (9-39) (C), as quantified by AUC analysis (D). N values for db/db mice are: eight vehicle-treated; eight GTS-21–treated; seven exendin (9-39)-treated; and seven GTS-21/exendin (9-39)-treated. E-I, ELISA of blood samples showed significantly increased levels of glucagon-like peptide-1 (GLP-1) (E), glucose-dependent insulinotropic peptide (GIP) (F), PYY1-36 (G), glucagon (H) and insulin (I) in db/db mice administered GTS-21, sitagliptin, or GTS-21 plus sitagliptin. For these assays, sitagliptin (10 mg/kg, BID) or vehicle control (saline, BID) was administered to db/db for 2 days by oral gavage. On day 3, overnight-fasted mice were administered GTS-21 (4 mg/kg, IP) or saline (IP) as a single dose, followed 30 minutes later by oral glucose (2 g/kg). After an additional 30 minutes, blood samples were obtained. Findings for GLP-1, GIP, PYY1-36, glucagon, and insulin were obtained using 102 male and female db/db mice. For all panels, the values are mean ± SEM with accompanying box and whiskers plots. **P* < .05 and ***P* < .01. NS, not significant; Sit./Sitaglip., sitagliptin. See Table S3 for detailed information concerning the numbers of male and female mice, mean body weights, and body weight ranges for these mice

3.6 | GTS-21 fails to improve glucose tolerance in α7nAChR and GLP-1R KO mice

To obtain target validation that α7nAChR mediates the action of GTS-21 to improve glucose tolerance, we evaluated GTS-21 glucoregulatory action in α7nAChR (−/−) global KO mice maintained on the C57BL/6J genetic background. GLP-1R (−/−) global KO mice maintained on the C57BL/6J genetic background were also evaluated to determine if the glucoregulatory action of GTS-21 is conditional on GLP-1R expression. For these studies, wild-type (WT) C57BL/6J mice of comparable age and body weight served as the reference strain. Also, when evaluating the glucoregulatory action GTS-21, the GLP-1R agonist exendin-4 served as a positive control.

In studies using the OGTT, single-dose GTS-21 (4 mg/kg, IP) lowered levels of blood glucose in WT mice, an effect that was significant in comparison with administered saline (Figure 4A,B). This action of GTS-21 was reproduced by single-dose exendin-4 (100 μg/kg, IP) that exerted a significantly larger effect (Figure 4A,B). However, in α7nAChR (−/−) KO mice the blood glucose-lowering action of GTS-21 was absent, whereas the action of exendin-4 was preserved (Figure 4C,D). In addition, the glucoregulatory actions of GTS-21 and exendin-4 were both absent in GLP-1R (−/−) KO mice (Figure 4E,F). Collectively, these findings provide evidence for a blood glucose-

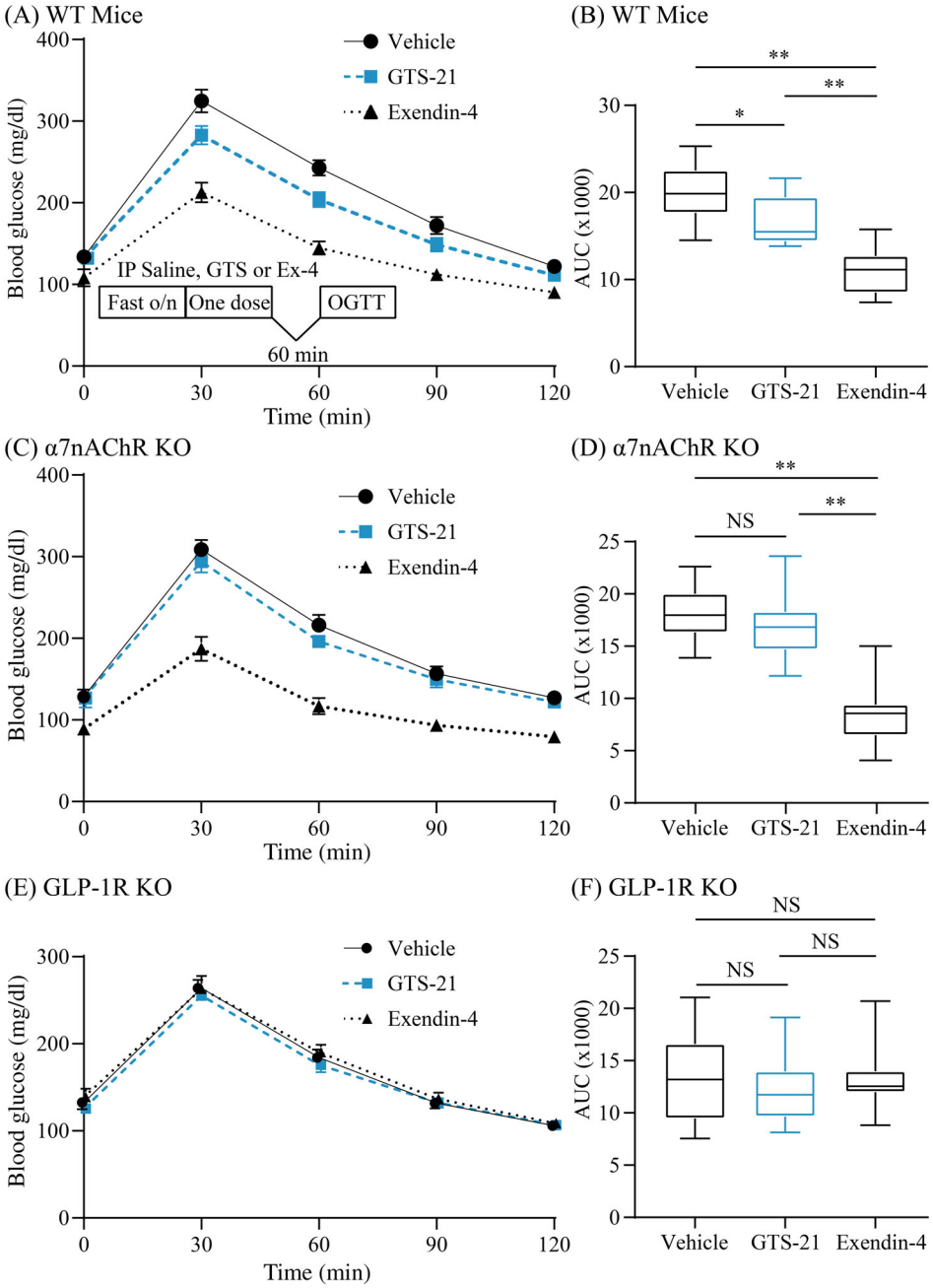

FIGURE 4 GTS-21 glucoregulatory action is lost in α7nAChR KO and glucagon-like peptide-1 receptor (GLP-1R) knockout (KO) mice. A and B, Control C57BL6/J mice were administered single-dose GTS-21 (4 mg/kg, IP), exendin-4 (100 μg/kg, IP), or vehicle control (saline, IP) after overnight fasting. Sixty minutes later, glucose (2 g/kg) was administered by oral gavage for the oral glucose tolerance test (OGTT). GTS-21 or exendin-4 alone significantly reduced blood glucose levels (A), as quantified by AUC analysis (B). N values for C57BL/6J mice are: ten vehicle-treated; 11 GTS-21–treated; and 11 exendin-4 treated. C and D, Same experimental design as for A and B, except that the OGTT was performed using α7nAChR KO mice maintained on the C57BL/6J genetic background. No significant glucoregulatory action of GTS-21 was measurable, whereas exendin-4 retained its blood glucose-lowering effect (C), as quantified by AUC analysis (D). N values for α7nAChR KO mice are: nine vehicle-treated; 10 GTS-21–treated; and nine exendin-4–treated. E and F, Same experimental design as for A and B, except that the OGTT was performed using GLP-1R KO mice maintained on the C57BL/6J genetic background. No significant glucoregulatory actions of GTS-21 or exendin-4 were measurable (E), as quantified by AUC analysis (F). N values for GLP-1R KO mice are: 13 vehicle-treated; 14 GTS-21–treated; and 11 exendin-4–treated. For all panels, the values are mean ± SEM with accompanying box and whiskers plots. *$P < .05$ and **$P < .01$. NS, not significant. See Table S4 for detailed information concerning the numbers of male and female mice, mean body weights, and body weight ranges for these mice

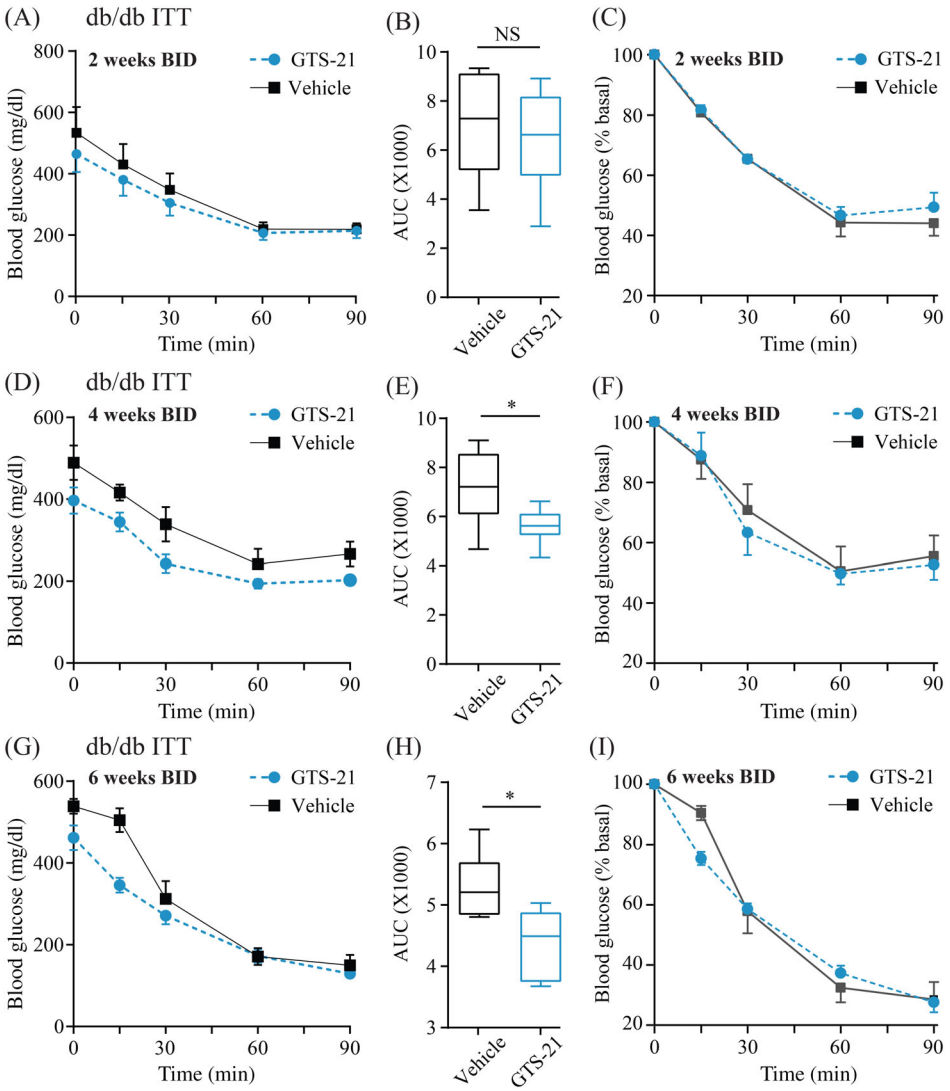

FIGURE 5 Multiple dosing with GTS-21 fails to increase insulin sensitivity in the insulin tolerance test (ITT). A-I, db/db mice were administered multiple-dose saline vehicle solution or GTS-21 (4 mg/kg, IP, BID) for A-C, 2, D-F, 4, or G-I, 6 weeks. On the morning of each experiment, an additional IP dose of GTS-21 (4 mg/kg) was administered. Mice were then fasted for 6 hours. Next, tail blood samples were obtained at time 0 minutes for determination of fasting blood glucose levels. Insulin (1 IU/kg, IP) was then administered, and additional blood was collected for determination of glucose levels at 15, 30, 60, and 90 minutes. Note that at time 0, mice administered GTS-21 exhibited a trend toward reduced fasting blood glucose levels (A-C). However, this action did not reach statistical significance in comparison with control mice receiving the vehicle saline solution (2 weeks: $P = .509$; 4 weeks: $P = .113$; 6 weeks: $P = .052$). B, E, H, AUC analysis revealed an 'apparent' increase of insulin sensitivity for 4- and 6-week GTS-21–treated mice (B, not significant, NS; E, $^*P < .05$; H, $^*P < .05$). C, F, I, However, when these glucose levels measured in the ITT were normalized relative to the initial fasting glucose level, no increase of insulin sensitivity was measured for mice administered GTS-21. N values for db/db mice are: six for 2-week vehicle-treated; seven for 2-week GTS-21–treated; six for 4-week vehicle-treated; six for 4-week GTS-21–treated; six for 6-week vehicle-treated; six for 6-week GTS-21–treated. For all panels, the values are mean ± SEM with accompanying box and whiskers plots. See Table S5 for detailed information concerning the numbers of male and female mice, mean body weights, and body weight ranges for these mice

lowering action of GTS-21 that is conditional on α7nAChR and GLP-1R expression.

3.7 | GTS-21 fails to increase insulin sensitivity or beta cell mass in db/db mice

To test if GTS-21 increases insulin sensitivity, as measured in an insulin tolerance test, mice were administered saline or GTS-21 (4 mg/kg, IP, BID) for 2, 4, or 6 weeks. An additional single dose of GTS-21 (4 mg/kg) was administered on the day of the experiment, after which mice were fasted for 6 hours. This protocol led to small reductions of fasting blood glucose measured at time 0 minutes prior to administration of insulin (Figure 5A,D,G). Next, insulin (1 IU/kg, IP) was administered so that blood glucose levels could be measured at the 15-, 30-, 60-, and 90-minute time points. AUC analysis revealed no change of insulin sensitivity using the 2-week dosing protocol, whereas an apparent increase was detected using

the 4- and 6-week protocols (Figure 5B,E,H). However, when correcting for the GTS-21–induced shift of fasting blood glucose levels, no change of insulin sensitivity was measured for any of the dosing protocols (Figure 5C,F,I). Next, to test for alterations of beta cell mass in response to GTS-21, db/db mice received GTS-21 (4 mg/kg, IP, BID) for 8 weeks. No significant action of GTS-21 was detected in assays that monitored insulin-positive surface area or islet fraction in pancreas slices evaluated by immunohistochemistry (Figure S3).

4 | DISCUSSION

We report that GTS-21 lowers levels of blood glucose in obese, hyperglycaemic, leptin receptor-deficient db/db mice, a model system for drug discovery relevant to the treatment of obesity and T2D.[11] Target validation studies using α7nAChR KO mice provide clear evidence that this action of GTS-21 is conditional on α7nAChR expression, and that the α7nAChR agonist PNU-282987 replicates the action of GTS-21. Remarkably, the glucoregulatory action of GTS-21 is missing in GLP-1R KO mice, is enhanced by the DPP-4 inhibitor sitagliptin, and is counteracted by GLP-1R antagonist exendin (9-39). Because GTS-21 also raises levels of circulating GLP-1 while failing to increase insulin sensitivity, we propose that GTS-21 engages the GLP-1 incretin hormone axis to stimulate pancreatic insulin secretion, thereby lowering blood glucose levels.

It must be pointed out that the precise concentration of circulating bioactive GLP-1 (7-36)amide measured in mice treated with GTS-21 remains unknown because of its fast hydrolysis and inactivation by DPP-4 and neutral endopeptidases in blood samples.[18] Furthermore, the half-life of GLP-1 is so short that its concentration in the circulation may be limiting for full GLP-1R stimulation.[19] Thus it is noteworthy that GTS-21 also raises circulating levels of GIP and glucagon as expected if GTS-21 stimulates α7nAChR located not only on intestinal L cells, but also on K cells and islet alpha cells. Because α7nAChR might be expressed on intermediary intestinal cell types that release paracrine factors (e.g. α-MSH) important to GLP-1 secretion,[20] indirect actions of GTS-21 mediated by these factors must also be taken into account. Intriguingly, it is now understood that islet alpha cells secrete not only glucagon, but also GLP-1 under conditions of stress, as might occur in db/db mice.[21] Because glucagon and GLP-1 are both beta cell GLP-1R agonists,[22,23] they might act as intra-islet paracrine hormones to mediate the insulin secretagogue and blood glucose-lowering actions of GTS-21.

Another possible explanation for the findings reported here is that GTS-21 acts at L cells to stimulate GLP-1 release, whereafter GLP-1 stimulates the GLP-1R on sensory vagus neurons located in close proximity to L cells in the intestinal wall.[24] Resultant excitation of these sensory neurons provides synaptic inputs to brainstem metabolic control centres to influence glycaemia, while also initiating vagovagal reflexes that are important to glucose homeostasis.[4,24-26] Importantly, vagovagal reflexes also initiate a cholinergic anti-inflammatory reflex (CAIR), in which ACh released from parasympathetic neurons stimulates α7nAChR on immune system cells,[27-32] thereby downregulating inflammatory cytokine production in adipocytes.[33] Such findings indicate that α7nAChR agonists exert a dual effect in that they improve glucoregulation while also combating adipose inflammation, as occurs in the metabolic syndrome.[5] Thus an opportunity exists to determine if disrupted crosstalk of neuronal CAIR and GLP-1 incretin hormone action contributes to the pathogenesis of T2D.

One prior study reported a prosurvival action of PNU-282987 to reduce beta cell apoptosis and to preserve beta cell mass in a mouse model of multiple low-dose streptozotocin-induced type 1 diabetes (T1D).[34] However, in the present study of db/db mice, we failed to observe any action of GTS-21 to alter beta cell mass. This discrepancy might be explained by a systems physiology difference when testing T1D model mice in which there is beta cell death[34] versus the db/db model that exhibits a compensatory increase of beta cell mass at a young age.[35]

In summary, this study provides a rationale to investigate if α7nAChR agonists administered in combination with a DDP-4 inhibitor such as sitagliptin might serve as a new treatment for T2D. Importantly, it remains to be determined whether α7nAChR agonists such as GTS-21 emulate the beneficial antidiabetogenic actions of synthetic GLP-1R agonists such as liraglutide, semaglutide, and dulaglutide in patients with T2D.[36-38]

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CONFLICT OF INTEREST

All authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Qinghe Meng, Oleg G. Chepurny, Colin A. Leech, Napat Pruekprasert, Megan E. Molnar, James Jason Collier, Robert N. Cooney, and George G. Holz generated and helped interpret experimental data while also contributing to the writing and editing of this manuscript. Qinghe Meng and Colin A. Leech prepared the figures. Robert N. Cooney, and George G. Holz are the guarantors of this work and, as such, had full access to all the data in this study, and both take responsibility for the integrity of data and the accuracy of data analysis.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/dom.14693.

DATA AVAILABILITY STATEMENT

Data will be available on request.

ORCID

Colin A. Leech https://orcid.org/0000-0002-5859-1304
George G. Holz https://orcid.org/0000-0002-1781-3580

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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