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ORIGINAL ARTICLE

A vitamin B12 conjugate of exendin-4 improves glucose tolerance without associated nausea or hypophagia in rodents

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Aims: While pharmacological glucagon-like peptide-1 receptor (GLP-1R) agonists are FDA-approved for treating type 2 diabetes mellitus (T2DM) and obesity, a major side effect is nausea/malaise. We recently developed a conjugate of vitamin B12 (B12) bound to the GLP-1R agonist exendin-4 (Ex4), which displays enhanced proteolytic stability and retention of GLP-1R agonism. Here, we evaluate whether the conjugate (B12-Ex4) can improve glucose tolerance without producing anorexia and malaise.

Materials and methods: We evaluated the effects of systemic B12-Ex4 and unconjugated Ex4 on food intake and body weight change, oral glucose tolerance and nausea/malaise in male rats, and on intraperitoneal glucose tolerance in mice. To evaluate whether differences in the profile of effects of B12-Ex4 vs unconjugated Ex4 are the result of altered CNS penetrance, rats received systemic injections of fluorescein-Ex4 (Flex), Cy5-B12 or Cy5-B12-Ex4 and brain penetrance was evaluated using confocal microscopy. Uptake of systemically administered Cy5-B12-Ex4 in insulin-containing pancreatic beta cells was also examined.

Results: B12-Ex4 conjugate improves glucose tolerance, but does not elicit the malaise and anorexia produced by unconjugated Ex4. While Flex robustly penetrates into the brain (dorsal vagal complex, paraventricular hypothalamus), Cy5-B12 and Cy5-B12-Ex4 fluorescence were not observed centrally, supporting an absence of CNS penetrance, in line with observed reduction in CNS-associated Ex4 side effects. Cy5-B12-Ex4 colocalizes with insulin in the pancreas, suggesting direct pancreatic action as a potential mechanism underlying the hypoglycaemic effects of B12-Ex4.

Conclusion: These novel findings highlight the potential clinical utility of B12-Ex4 conjugates as possible future T2DM therapeutics with reduced incidence of adverse effects.

KEYWORDS

antidiabetic drug, appetite control, drug development, exenatide

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1 | INTRODUCTION

Multiple incretin-based therapeutics are approved for the treatment of type 2 diabetes mellitus (T2DM) because of their ability to elicit pancreatic insulin secretion and reduce blood glucose levels. These pharmacotherapies include compounds designed to increase endogenous concentrations of the incretin hormone glucagon-like peptide-1 (GLP-1) by inhibiting the endopeptidase DPP-IV, as well as synthetic peptide-based GLP-1 receptor (GLP-1R) agonists resistant to DPP-IV degradation.[1] In addition to being the more potent class of GLP-1-based therapeutics for reducing glycaemia, GLP-1R agonists significantly reduce food intake and body weight in both humans and animal models.[1-3] This anorectic effect is attractive when considering the utilization of GLP-1R agonists as an on- or off-label treatment option for obesity, and indeed, the GLP-1R agonist liraglutide is FDA-approved for the treatment of obesity.[4] However, a sizeable percentage of individuals with T2DM does not have obesity or overweight[5-7] and may want to avoid weight loss. Furthermore, it is important to note that the hypophagic effects of GLP-1R agonists are accompanied by a pronounced incidence of nausea, vomiting and malaise.[3,8,9] In fact, ~20% to 50% of T2DM patients receiving GLP-1-based medication experience nausea and/or vomiting, leading to discontinuation of drug treatment in ~6% to 10% and reduced dose tolerance in another ~15%.[10-14] These adverse effects are surprisingly under-investigated, as they limit the widespread use, efficacy and potential ubiquitous utility of GLP-1R agonists (eg, liraglutide, exenatide) for the treatment of metabolic disease.

A wealth of literature provides convincing evidence that a significant portion of the increase in glucose-stimulated insulin secretion following exogenous GLP-1R ligand administration is mediated by direct activation of GLP-1R expressed on pancreatic β-cells (see References 1–3,15 for review), mimicking the paracrine effects of pancreatic-derived GLP-1 that may not occur with endogenous L-cell-derived GLP-1.[16-18] Importantly, activation of GLP-1R expressed on vagal afferent fibers and/or in discrete nuclei in the central nervous system (CNS) also contributes to exogenous GLP-1R agonist-mediated improvements in glycaemic control.[1-3,15,19-21] Interestingly, these dual sites of action (ie, vagal and direct CNS activation) also mediate the intake- and body weight-suppressive effects of exogenous systemic GLP-1R agonist administration.[21-23] Both liraglutide and exenatide can penetrate into the CNS and activate central GLP-1R-expressing nuclei, leading to hypophagia and weight loss.[22,24] Remarkably, however, GLP-1R agonist-induced illness behaviors (eg, nausea, conditioned taste avoidance, hypophagia, emesis) are mediated by GLP-1Rs expressed within the CNS and not by vagal afferent GLP-1Rs when these compounds are delivered systemically.[8] Although other T2DM medications (eg, DPP-IV inhibitors) may improve glycaemic control with minimal effects on energy balance,[25,26] long-lasting GLP-1R agonists provide several advantages, such as improved glycaemic control and less frequent administration.[27-29] Thus, from a therapeutic standpoint, designing a novel GLP-1R agonist that is resistant to DPP-IV degradation, that does not penetrate readily into the CNS, but retains the enhanced pharmacokinetic and pharmacodynamic profile of such agonists on pancreatic β-cells would theoretically provide a new, improved pharmacological tool for glycaemic control in T2DM patients without eliciting unwanted hypophagia and nausea.

Recently, we demonstrated that covalent conjugation of the GLP-1R agonist exendin-4 (Ex4) to vitamin B12 (cyanocobalamin) (B12) between the vitamin 5′-OH group and the K12 position of Ex4 retains picomolar agonism (68 pM)[30] of the GLP-1R, either as the free conjugate (B12-Ex4 as used in this study) (Figure 1) or bound to Intrinsic Factor (IF; 126 pM),[30] a B12 transport protein critical for B12 absorption in humans. This work also confirmed that IF bound B12-Ex4 with low nanomolar affinity, as occurs with cyanocobalamin.[30]

Interestingly, while Ex4 readily penetrates the CNS,[22,31] little is known about the penetrance of B12 in the brain. Uptake of B12 into the brain is putatively a receptor-mediated process with megalin, a receptor capable of TCII-B12 uptake in the kidney, for example, being expressed in the choroid plexus.[32,33] Additional evidence points to the importance of the CD320 receptor, as genetic ablation in mice results in severe cobalamin deficiency in the mouse brain,[34,35] as well as the transmembrane protein amnionless, natural mutations of which result in Imerslund-Gräsbeck syndrome and congenital cobalamin malabsorption.[36] Collectively, this information points to a receptor-mediated process of B12 blood-brain barrier penetrance, but little is known of where B12 is transported in the brain, and to what extent,[37] relative to total concentrations. It is evident that CNS uptake is considerably lower compared to other organs, especially the liver and kidney,[38,39] with one recent study, using a B12-[89]Zr PET probe, revealing less than 0.1% injected dose per gram (ID/g) in brain in mouse models with over 5% ID/g observed in pancreas.[40] We therefore hypothesized that a B12-Ex4 conjugate would retain the incretin profile of a GLP-1R agonist to improve glucose tolerance, but would prevent development of nausea/malaise through reduced, or redirected, CNS/hypothalamic penetration of the agonist.

The rat is a unique animal model for pre-clinical testing of the B12-Ex4 conjugate. Rats, unlike humans and mice, demonstrate an unexpected hyperglycaemic response to systemic Ex4 delivery. This hyperglycaemic effect is unique to the Ex4 molecule (among approved GLP-1R agonists) in the rat and is explained, in part, by CNS-mediated sympathetic activation.[41] Further, rats show well-documented hypophagic effects to GLP-1R ligands, mediated partly by accompanying acute effects on nausea/malaise, similar to humans, but not to mice.[8,9] Rats were therefore used as the primary model to evaluate the effects of B12-Ex4 on glycaemic control, energy balance and nausea/malaise, and these effects were compared with the
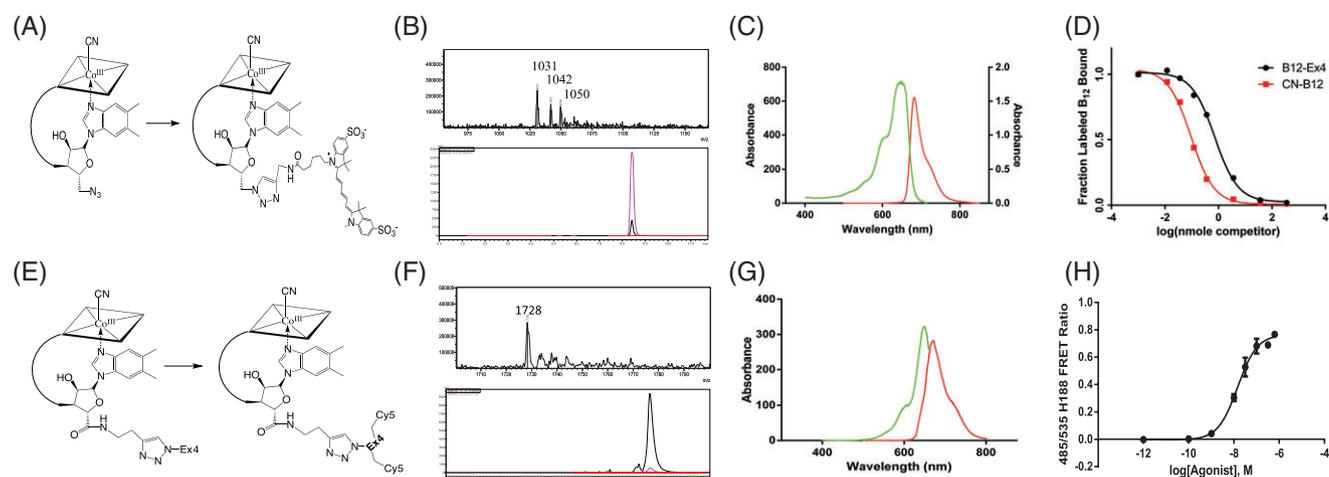
FIGURE 1 A, Synthetic scheme for Cy5-B12, Cy5 alkyne was "clicked" onto a B12-azide conjugate. B, RP-HPLC of Cy5-B12 showing purity ≥98% and LC-MS showing $1031[M + 2H]^{+2}$, $1042[M + Na + 2H]^{+2}$ and $1050[M + K + 2H]^{+2}$, consistent with the conjugate. C, Excitation and emission spectra of Cy5-B12 at 645 and 682 nm, respectively. D, Human recombinant TCII binding of B12-Ex4 and cyano-B12 with a K_d of 0.75 and 0.98 nM, respectively. E, Synthetic scheme for Cy5-B12-Ex4, Cy5-NHS ester was conjugated to Ex4's lysine 26 and N-terminal. F, RP-HPLC of Cy5-B12-Ex4 showing purity ≥91% and LC-MS showing m/z = $1728 [M + 4H]^{+4}$, consistent with conjugate containing 2 molecules of Cy5 per B12-Ex4 component. G, Excitation and emission spectra of Cy5-B12-Ex4 at 648 and 670 nm, respectively. H, Cy5-B12-Ex4 agonism at the GLP-1 receptor reported using the FRET reporter H188; EC_{50} = 13 nM. Note that B12-Ex4 agonism at the GLP-1 receptor was previously reported (68 pM)[30]

response profile after peripheral administration of unconjugated Ex4. Given that Ex4 produces hypoglycaemic effects in mice,[42,43] similar to the effect observed in humans,[44,45] blood glucose levels in mice were assessed also in a glucose tolerance test (GTT) upon B12-Ex4 or Ex4 administration. The data presented here provide evidence for second-generation "cobalaminylated" GLP-1R agonists for the treatment of T2DM, with a pronounced profile of effects that include glucoregulation without anorexia or body weight loss, and most critically, an absence of nausea/malaise.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult male Sprague Dawley rats (Charles River, Wilmington, Massachusetts) were singly housed in hanging wire mesh cages. Four-month old C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) were singly housed in plastic cages. All animals were housed under a 12-hour:12-hour light/dark cycle in a temperature- and humidity-controlled environment. Standard rodent chow (Purina 5001) and tap water were available ad libitum except where noted. Procedures were approved by the Institutional Care and Use Committee of the University of Pennsylvania.

2.2 | Compounds

B12-conjugated exendin-4 (B12-Ex4) was produced, characterized and screened for agonism at the GLP-1R (EC_{50} of 68 pM, relative to 28 pM for Ex4 in the same assay), as previously described.[30] One addition to the characterization was the measure of TCII binding of B12-Ex4 (Figure 1D), which was conducted at the Department of Clinical Medicine-Clinical Biochemistry, University of Aarhus, Denmark as

described previously.[46] B12-Ex4, Ex4 (Bachem, Torrance, California) and lithium chloride (LiCl) (Sigma Aldrich, St. Louis, Missouri) were dissolved in sterile 0.9% NaCl for peripheral injections. Injections were separated by at least 48 hours. For most in vivo experiments, injections were administered using a within-subjects, Latin square design. The exception was the conditioned taste avoidance (CTA) study, which used a between-subjects design.

2.3 | Effects of B12-Ex4 on energy balance

Shortly before onset of the dark phase, rats (n = 12) received subcutaneous (SC) injection of B12-Ex4 (1, 5 or 20 μg/kg) or vehicle (1 mL/kg sterile saline). Chow intake was measured at 1, 3, 6 and 24 hours post-injection. Food spillage was accounted for in all intake measurements. Body weight was also measured at 0 and 24 hours.

2.4 | Effects of B12-Ex4 on glycaemic control during oral glucose tolerance test (OGTT)

Rats (n = 12) were deprived of food overnight before testing. On the morning of testing, just after onset of the dark phase, water was also removed from the cage. A small drop of blood was collected from the tail tip and was analysed for blood glucose (BG) level using a standard glucometer (AccuCheck). Immediately after this baseline BG reading (t = −30 minutes), each rat received SC injection of B12-Ex4 (5 or 20 μg/kg) or vehicle (1 mL/kg sterile saline); doses of drug were selected based on results of the feeding study. BG was measured 30 minutes later (t = 0 minutes) and each rat received an oral gavage of glucose (2 g/kg). Subsequent BG readings were taken at 20, 40, 60 and 120 minutes after glucose gavage. After the final BG reading, food and water were returned.

2.5 | Effects of systemic Ex4 on glycaemic control and energy balance

The effects of unconjugated Ex4 were evaluated in an OGTT, using methods similar to those described above, with 2 major differences: SC injections were Ex4 (5 or 20 μg/kg) or vehicle (1 mL/kg sterile saline), and food intake and body weight change after completion of the OGTT were monitored. Pre-weighed food was returned to the rats after the OGTT, and chow intake was measured for ~21.5 hours (eg, until 24 hours after the SC injections). Spillage was accounted for in food intake measurements. Body weight was recorded at 0 and 24 hours. For the OGTT, rats (n = 10) were tested; food and body weight data were collected with 1 less rat included (n = 9) following a technical error in food intake measurement.

2.6 | Effects of B12-Ex4 on expression of a conditioned taste avoidance (CTA)

Rats (n = 8–10 per drug) were evaluated for expression of a CTA to a flavour paired with B12-Ex4 (5 μg/kg, IP). Ex4 (5 μg/kg, IP) and LiCl (0.15 M) were used as positive controls. A 2-bottle test was used so each rat had access to a flavour that had been paired previously with vehicle (1 mL/kg saline, IP) and a flavour that had been paired previously with drug (B12-Ex4, Ex4 or LiCl). See Appendix S1 for more details.

2.7 | Effect of B12-Ex4 on glycaemic control in mice during intraperitoneal glucose tolerance test (IPGTT)

The experimental procedure for IPGTT in mice was similar to that used for OGTT in rats. Briefly, mice (n = 13; 8 females, 5 males) were food- and water-deprived for 4 hours before and during the IPGTT. Testing was completed at the mid-light phase. Blood was collected from the tail tip and was analysed for BG. Immediately after this baseline reading (t = −30 minutes), each mouse received IP injection of Ex4 (5 μg/kg), B12-Ex4 (equimolar dose to Ex4) or saline (10 μL/g). BG was measured 30 minutes later (t = 0 minutes) and each mouse received IP injection of glucose (2 g/kg). Subsequent BG readings were taken at 20, 40, 60 and 120 minutes after glucose injection. After the final BG reading, food and water were returned. Area under the curve (AUC) was calculated from 0 to 120 minutes (eg, beginning at the time of glucose administration) using the trapezoidal method.

2.8 | B12-Exendin-4-Cyanine-5 (Cy5-B12-Ex4) synthesis

B12-Ex4 was synthesized as described previously.[30] B12-Ex4 (0.5 mg, 0.0001 mmol) was dissolved in PBS buffer pH 7.6 (450 μL) and sulfo-cyanine5-NHS-ester (1 mg, 0.001 mmol) (Lumiprobe) was added (in 50 μL DMSO). The resulting solution was allowed to mix for 2 hours at room temperature, protected from light, and was then purified through RP-HPLC on a Shimadzu Prominence HPLC using a C18 column (Eclipse XDB-C18 5 μm, 4.6 × 150 mm). Solvents: A: 0.1% TFA water and B: Acetonitrile. Method: B%: 1% to 70% over 15 min. t_R: 12.1 minutes. Yield: 98%. Emission and excitation were 648 and 670 nm, respectively, using a Varian Cary UV Spectrophotometer and Agilent Cary Eclipse Fluorescence Spectrophotometer

(Agilent, Santa Clara, California), solvent H_2O/MeCN. LC-MS analysis (Shimadzu LCMS-8040, Method: 0.1% formic acid and 35% methanol water at 0.2 mL/min, DL temp: 150°C, heat block temp: 400°C.): expected m/z: 6923 [B12-Ex4-(Cy5)$_2$], observed: 1383 [M + 5H]$^{+5}$, 1728 [M + 4H]$^{+4}$. See Figure 1 for more information.

2.9 | B12-Cyanine-5 (Cy5-B12) synthesis

Cy5-B12 was synthesized using Huisgen/Sharpless "Click" Chemistry.[47,48] Cu(I) (1 mg, 0.005 mmol) and Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (3.5 mg, 0.006 mmol) and were dissolved in 0.5 mL DMF/H2O (4:1 v/v). Once colour change occurred, the previously synthesized B12-Azide (3 mg, 0.002 mmol)[49] and Cyanine-5 alkyne (0.5 mg, 0.0007 mmol) (Lumiprobe Hunt Valley, Maryland) were dissolved in the solution and allowed to stir at room temperature overnight, protected from light. This was purified through RP-HPLC on a Shimadzu Prominence HPLC using a C18 column (Eclipse XDB-C18 5 μm, 4.6 × 150 mm). Solvents: A: 0.1% TFA water and B: Acetonitrile. Method: B%: 20% to 72% over 18 minutes. t_R: 4.7 min. Yield: 94%. LC-MS analysis (Shimadzu LCMS-8040, Method: 0.1% Formic acid and 35% methanol water at 0.2 mL/min, DL temp: 150°C, heat block temp: 400°C.): expected m/z: 2059 observed: 1031 [M + 2H]$^{+2}$, 1042 [M + Na + 2H]$^{+2}$, and 1050 [M + K + 2H]$^{+2}$. Emission and excitation were 645 and 682 nm, respectively, using a Varian Cary UV Spectrophotometer and Agilent Cary Eclipse Fluorescence Spectrophotometer, solvent H_2O. See Figure 1 for more information.

2.10 | GLP-1 assay for Cy5-B12-Ex4

Agonism at the GLP-1 receptor was monitored using HEK-293 cells stably transfected with the GLP-1 receptor cultured in DMEM with 10% FBS, 1% pen/strep and 250 μg/mL geneticin/g-418. Cells were plated on a rat-tail-collagen-coated 96-well plate at 60 000 cell/well and were allowed to adhere overnight. Cells were infected with an adenovirus to express the H188 FRET reporter, using a 25 MOI for 16 to 20 hours in 75 μL of DMEM-1% FBS. After viral incubation, cells were placed in 200 μL standard extracellular matrix with glucose and 0.1% BSA. Conjugates were added to each well at 5× the required concentration. Agonism was determined through an increase in 485/553 nm FRET ratio, indicative of an increase in cAMP level through cAMP binding to an exchange protein directly activated by cAMP (EPAC).[50]

2.11 | Immunohistochemical procedures and confocal imaging

Rats (n = 4/group) received IP injection of fluorophore-labeled Ex-4 (Flex; 5 μg/kg; 0.0001 nM; AnaSpec[51]), Cy5-B12-Ex4 (5 μg/kg; 0.03 nM), Cy5-B12 (5 μg/kg) or Cy5-B12-Ex4 delivered at a dose equimolar to Flex (0.0001 nM). Rats were transcardially perfused 3 hours after injection, using 0.1 M PBS, followed by 4% paraformaldehyde (PFA). Brains were collected and sections from the area postrema and hypothalamus were processed via immunohistochemistry for NeuN and GFAP, mounted and coverslipped with DAPI mounting

medium. Sections were visualized via confocal microscopy. See Appendix S1 for more detail.

To evaluate penetrance of B12-Ex4 in the pancreas, rats (n = 2) were given IP injection of Cy5-B12-Ex4 (5 μg/kg) and were transcardially perfused 3 hours later with 4% paraformaldehyde in PBS. Pancreases were collected and sagittally sectioned, processed via immunohistochemistry for insulin and coverslipped with DAPI mounting medium. Sections were visualized with confocal microscopy and 3-dimensional rotational animations were rendered from the collected z-stack images using Imaris 8.1.2 (Bitplane, Zurich, Switzerland). See Appendix S1 for more detail.

2.12 | Statistical analyses

See Appendix S1.

3 | RESULTS

3.1 | B12-Ex4 has potent beneficial effects on glycaemic control, but minimal impact on feeding and body weight in rats

Ex4 and other GLP-1R agonists reduce blood glucose levels, and are used clinically to treat T2DM.[52] In addition, the food intake- and body weight-suppressive effects of GLP-1R agonists have highlighted the utility of these pharmacotherapies for treatment of obesity.[53] To evaluate whether the metabolic effects of B12-Ex4 are similar to those of other GLP-1R agonists such as unconjugated Ex4, the effects of SC injection of B12-Ex4 on energy balance and glycaemic control were evaluated. To confirm TCII binding of B12 in its Ex4

conjugated form (ie, B12-Ex4), a radio chase assay using ^{57}Co-labelled B12 was conducted,[46] and confirmed low nanomolar binding (~75 nM) was maintained (Figure 1D).

First, to test whether B12-Ex4 has intake- and body weight-suppressive effects similar to those of Ex4, rats were given SC injection of B12-Ex4 (0, 1, 5 or 20 μg/kg in 1 mL/kg sterile saline), and subsequent food intake (1, 3, 6, 24 hours) and body weight gain were measured. The highest dose of B12-Ex4, 20 μg/kg, significantly suppressed food intake at 3 and 6 hours post injection (Figure 2A) (drug × time interaction, $F_{9,99}$ = 3.69, $P < .001$; 0 vs 20 μg/kg, $P < .05$ at 3 and 6 hours). No other significant effects on food intake were observed, at other times or with other doses of drug (all other $P > .05$). There was also no significant effect of B12-Ex4 on 24-hour body weight change (Figure 2B) ($F_{3,33}$ = 0.50, $P = .69$), which is consistent with the fact that cumulative 24-hour energy intake was similar among the treatment conditions.

Next, the glycaemic effects of B12-Ex4 (5 or 20 μg/kg) or vehicle (1 mL/kg sterile saline, SC) were evaluated via OGTT. B12-Ex4 significantly reduced blood glucose levels in the OGTT (Figure 2C) (main effect of drug, $F_{2,22}$ = 4.01, $P < .04$; drug × time interaction, $F_{10,110}$ = 17.29, $P < .000001$). Posthoc analyses showed that both doses of B12-Ex4 significantly suppressed BG at 20 and 40 minutes after glucose gavage (vs vehicle; all $P < .05$). A dose-responsive effect is also suggested by the finding that 20 μg/kg B12-Ex4 had more potent BG-suppressive effects than 5 μg/kg at 20 minutes after glucose gavage ($P < .05$). Interestingly, BG levels were increased by both doses of B12-Ex4 at 60 minutes and by the higher dose at 120 minutes (all $P < .05$). Importantly, injection of B12-Ex4 had no effect on blood glucose levels on its own (t = 0 minutes, all $P > .05$).

FIGURE 2 B12-Ex4 potently suppresses blood glucose in an oral glucose tolerance test but has minimal effects on energy balance control. Food intake and body weight change were measured after SC administration of B12-Ex4. A, Only the highest dose of drug, 20 μg/kg, produced any reduction in feeding. B, No changes in 24-hour body weight gain were observed as a result of B12-Ex4 administration. C, In a separate experiment, SC injection of B12-Ex4 (0 μg/kg indicated by white circles, 5 μg/kg by lighter blue circles, 20 μg/kg by darker blue circles) reduced blood glucose in an oral glucose tolerance test from 20 to 40 minutes after injection. *, significantly different from vehicle (P < .05); &, significantly different from 5 μg/kg (P < .05). Key in (A) also applies to (B). Data are given as mean ± SEM
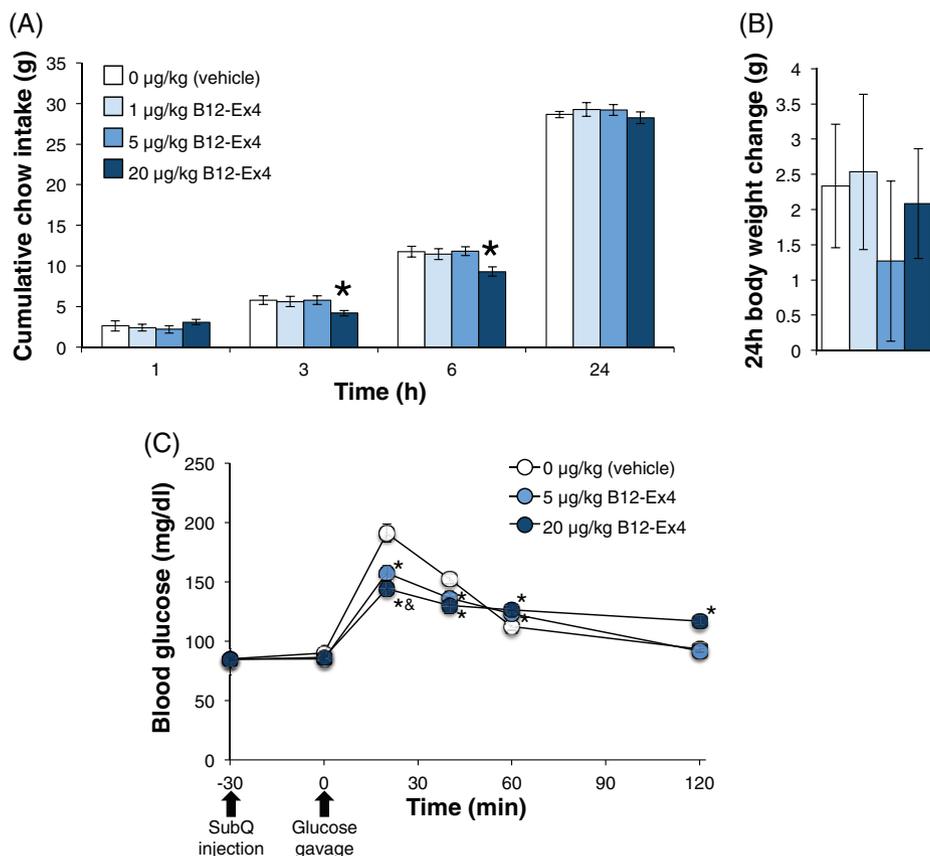
3.2 | Systemic injection of Ex4 produces hyperglycaemia, hypophagia and weight loss

The rat is a particularly interesting model to test the effects of an Ex4-based drug on glycaemic and energy balance control, because rats exhibit a hyperglycaemic response to acute peripheral administration of Ex4 as a result of sympathetic activation,[41] but they also show pronounced reductions in feeding and body weight gain.[54–56] To evaluate the effects of SC administration of unconjugated Ex4 on these measures, and to be able to more directly compare the effects of B12-Ex4 to those of Ex4, an OGTT was administered to rats after SC injection of Ex4 (5 or 20 μg/kg) or vehicle (1 mL/kg), and subsequent chow intake and body weight were monitored after the OGTT. Similar to previous findings,[41] systemic Ex4 produced a pronounced hyperglycaemic response in the rats (Figure 3A) (main effect of drug, $F_{2,18} = 8.84$, $P < .01$; drug × time interaction, $F_{10,90} = 11.89$, $P < .000001$). Injection of either dose of Ex4 increased BG on its own (eg, before administration of glucose; at t = 0 minutes, vehicle vs 5 or 20 μg/kg, $P < .05$). BG levels remained significantly elevated

in Ex4-treated rats at 40, 60 and 120 minutes after glucose gavage (vehicle vs 5 or 20 μg/kg, all $P < .05$).

When food was returned after the last BG reading, Ex4-treated rats ate significantly less than did vehicle-treated controls in the subsequent 21.5 hours (Figure 3B) ($F_{2,16} = 43.74$, $P < .000001$; vehicle vs 5 or 20 μg/kg, $P < .05$) and gained less body weight (Figure 3C) ($F_{2,16} = 8.31$, $P < .01$; vehicle vs 20 μg/kg, $P < .05$). These results demonstrate the unique constellation of effects produced by peripheral Ex4 administration in the rat and, more importantly, highlight the distinct differences between Ex4 and B12-Ex4 for glycaemic and energy balance control.

3.2.1 | Ex4 elicits expression of a robust CTA that is not observed with B12-Ex4

GLP-1R agonists such as Ex4 have undesired side effects including nausea/malaise.[8,52] To evaluate whether B12-Ex4 produces nausea/malaise, rats were evaluated for expression of a conditioned taste avoidance (CTA) to B12-Ex4 (5 μg/kg, IP). Additional groups of rats
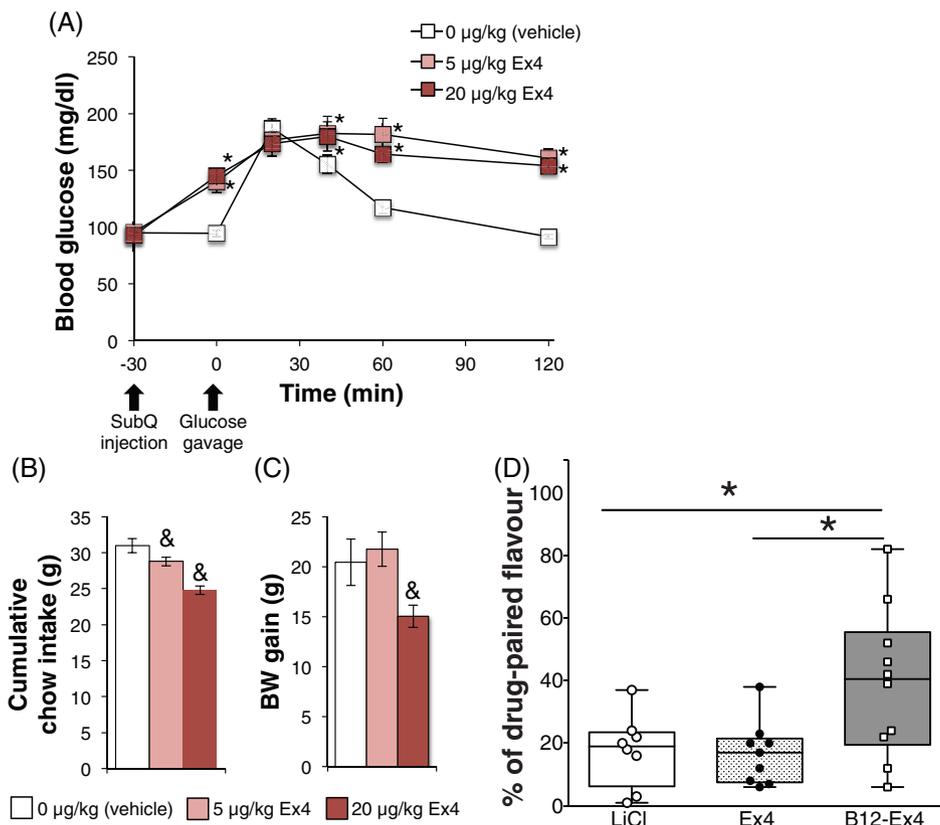
FIGURE 3 Systemic administration of Ex4 produces a different profile of metabolic effects than B12-Ex4. A, In contrast to the potent suppression of blood glucose produced by B12-Ex4, SC injection of Ex4 (0 μg/kg indicated by white squares, 5 μg/kg by lighter red squares, 20 μg/kg by darker red squares) produced a robust hyperglycaemic response. B, Food intake and C, body weight gain were suppressed by SC Ex4. To compare the induction of nausea/malaise by Ex4 with that potentially produced by B12-Ex4, rats were tested for expression of a conditioned taste avoidance (CTA) of a flavour paired with IP injection of B12-Ex4 (5 μg/kg), Ex4 (5 μg/kg) or LiCl as a positive control (0.15 M). D, The percent acceptance of the drug-paired flavour is shown as a box-and-whiskers plot. Both Ex4 (individual responses represented by black circles, overall group response represented in light gray box) and LiCl (individual responses represented by white circles, overall group response represented in white box) produce avoidance of the drug-paired flavour, as indicated by a reduced acceptance of the flavour. These effects are significantly different from acceptance of the drug-paired flavour in B12-Ex4-treated animals (individual responses represented by white squares, overall group response represented in dark gray box). A: *, significantly different from vehicle ($P < .05$); B, C: &, significantly different from all other groups ($P < .05$); D: *, significant difference between groups ($P < .05$). Key under (B) and (C) applies to both panels. Data in (A-C) are given as mean ± SEM

were evaluated in this experiment for CTA to Ex4 (5 μg/kg, IP) or to LiCl (0.15 M, IP), which is well known to produce nausea and CTA in rodents.[8,57,58] As shown in Figure 3D, acceptance of the drug-paired flavour was significantly higher in the B12-Ex4-treated group compared to either LiCl or Ex4 ($F_{2,24}$ = 5.29, P < .01; B12-Ex4 vs LiCl or Ex4, P < .05; LiCl vs Ex4, P > .05), suggesting that B12-Ex4 does not produce the same nausea/malaise as Ex4.

3.2.2 | In mice, B12-Ex4 and Ex4 suppress blood glucose levels in a glucose tolerance test

To confirm the ability of B12-Ex4 to improve glycaemic control in species that do not exhibit Ex4-induced stress-mediated hyperglycae-mic responses, the glycaemic effects of equimolar doses of B12-Ex4 and Ex4 were tested via IPGTT in mice. In contrast to rats, and more in line with human data,[44,45] Ex4 administration strongly attenuated the increase in blood glucose levels after IP glucose administration. Similarly, B12-Ex4 reduced blood glucose levels in the IPGTT (Figure 4A) (main effect of drug, $F_{2,24}$ = 67.13, P < .0001; drug × time interaction, $F_{10,120}$ = 15.07, P < .0001). Posthoc analyses showed that both compounds significantly suppressed BG at 20, 40, 60 and 120 minutes after glucose injection (all P < .05). Interestingly, injection of B12-Ex4 or Ex4 also reduced BG levels prior to IP glucose injection (t = 0, all P < .05). Although Ex4 had a more potent effect on BG at 20 minutes, compared to B12-Ex4 (P < .05), area under the curve analyses revealed that both compounds had hypogly-caemic effects post-glucose load compared to saline (Figure 4B) ($F_{2,24}$ = 62.74, P < .0001; vehicle vs B12-Ex4 or vs Ex4, P < .05).

### 3.2.3 | Unlike Ex4, B12-Ex4 does not readily penetrate into the CNS

Previous work shows that Ex4 crosses the blood-brain barrier to exert effects on energy balance and illness/malaise.[22,31,51] As B12-Ex4 treatment produces the glycaemic benefits associated with Ex4 without producing the centrally-mediated effects of hypophagia and nausea, this suggests that B12-Ex4 may be excluded from the CNS. To evaluate this possibility, rats were treated systemically with a fluorescent-tagged version of B12-Ex4 (Cy5-B12-Ex4), and pene-trance into the brain was evaluated using confocal microscopy. The results were compared with CNS penetrance of a fluorescent-tagged version of Ex4 (Flex), which has been shown to penetrate into the CNS,[51] and fluorescent-tagged B12 (Cy5-B12). The pres-ence of each of these fluorescent compounds was evaluated in the dorsal vagal complex (DVC) (Figure 5) and paraventricular nucleus of the hypothalamus (PVN) (Figure 6), because of the known impor-tance of these areas in mediating the feeding effects of GLP-1R activation[59,60] and the hyperglycaemic response observed in rats after systemic Ex4.[41] Consistent with previous data,[51] Flex was observed in the DVC, and was observed also within the PVN. In contrast, Cy5-B12 and Cy5-B12-Ex4 were not detected in either nucleus, suggesting that exogenously-injected B12 does not readily penetrate into these regions of the CNS and, hence, that conjuga-tion of B12 to Ex4 greatly reduces or prevents Ex4 from entering the same areas.

3.2.4 | B12-Ex4 is colocalized on insulin-producing pancreatic beta cells

The finding that peripherally administered B12-Ex4 is not detected in the DVC or PVN suggests that the glycaemic effects of the compound are probably mediated via peripheral actions. The pan-creas is a prime candidate for a peripheral site of action being responsible for the glycaemic-suppressive effects of B12-Ex4. Indeed, GLP-1R agonists can act directly on pancreatic beta cells to stimulate insulin release, thereby improving blood glucose levels.[61-63] To assess whether B12-Ex4 is taken up by insulin-producing pancreatic beta cells, rats were given systemic injection of Cy5-B12-Ex4 (5 μg/kg) and colocalization with insulin-expressing cells was analysed in the pancreas with 3-dimensional confocal microscopy. Results show robust colocalization of Cy5-B12-Ex4 with insulin in pancreatic sections (Figure 7) (Videos S1 and S2), supporting the hypothesis that B12-Ex4 acts at the pancreas to improve glycaemic control.

FIGURE 4 Systemic administration of B12-Ex4 or Ex4 suppresses blood glucose in mice. A, In an intraperitoneal glucose tolerance test, Ex4 (5 μg/kg) and B12-Ex4 (dose equimolar to Ex4) suppressed blood glucose levels prior to (t = 0 minutes) and after (t = 20, 40, 60, 120 minutes) IP glucose administration; vehicle vs B12-Ex4: **P < .01, ***P < .001; vehicle vs Ex4: ###P < .001; B12-Ex4 vs Ex4: §§P < .01. B, Area under the curve analyses from 0 to 120 minutes (ie, post-glucose load) show that B12-Ex4 (blue) and Ex4 (red) reduce AUC compared to saline vehicle (white); *P < .05, ***P < .001. Data are given as mean ± SEM
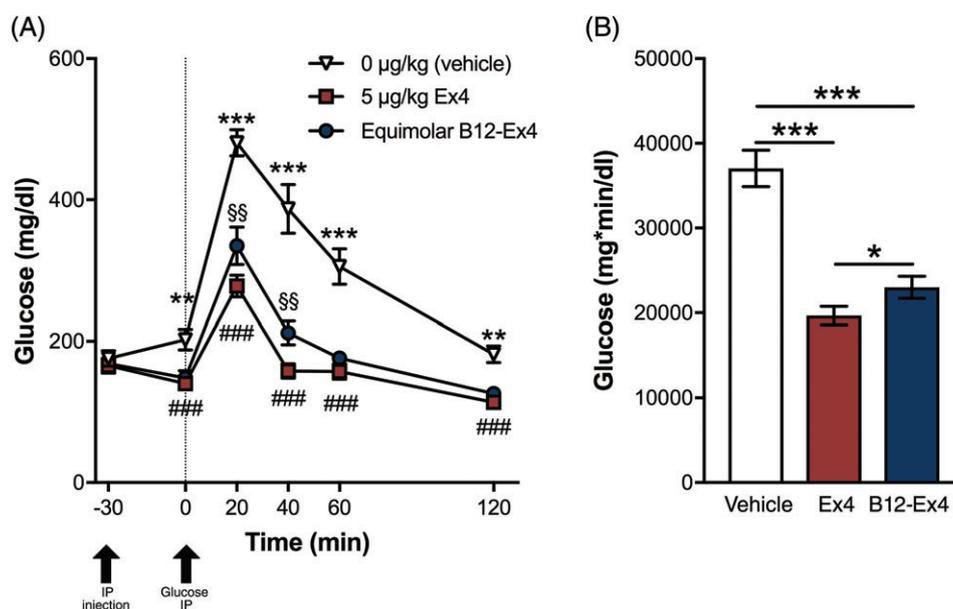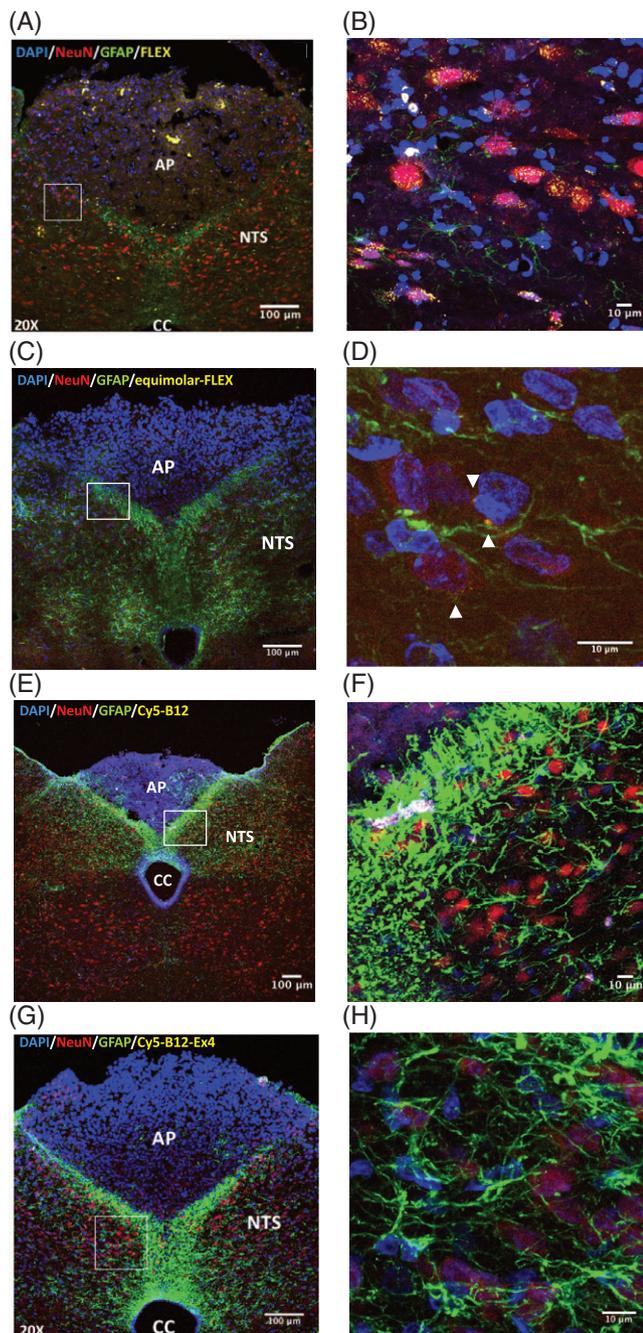
FIGURE 5 Systemically-delivered fluorescently labeled Ex-4 (Flex) highly penetrates within the DVC, whereas Cy5-B12 and Cy5-B12-Ex4 do not. A, C, E, G, Images were acquired at 10 to 20× or B, D, F, H, 63× (with 2-3× optical zoom) magnifications. Brains were processed for immunohistochemistry to label Flex, Equimolar-Flex, Cy5-B12 and Cy5-B12-Ex4 (yellow), astrocytes (GFAP) (green) and neurons (NeuN) (red). Sections were counterstained using DAPI (blue) to visualize cell nuclei. B, Flex and D, equimolar-Flex immunoreactivity is readily visualized in neurons and astrocytes in the DVC. F, Cy5-B12 and H, Cy5-B12-Ex4 are not present either in neurons or in astrocytes within the DVC. Abbreviations: AP, area postrema; CC, central canal; DVC, dorsal vagal complex; NTS, nucleus tractus solitarius
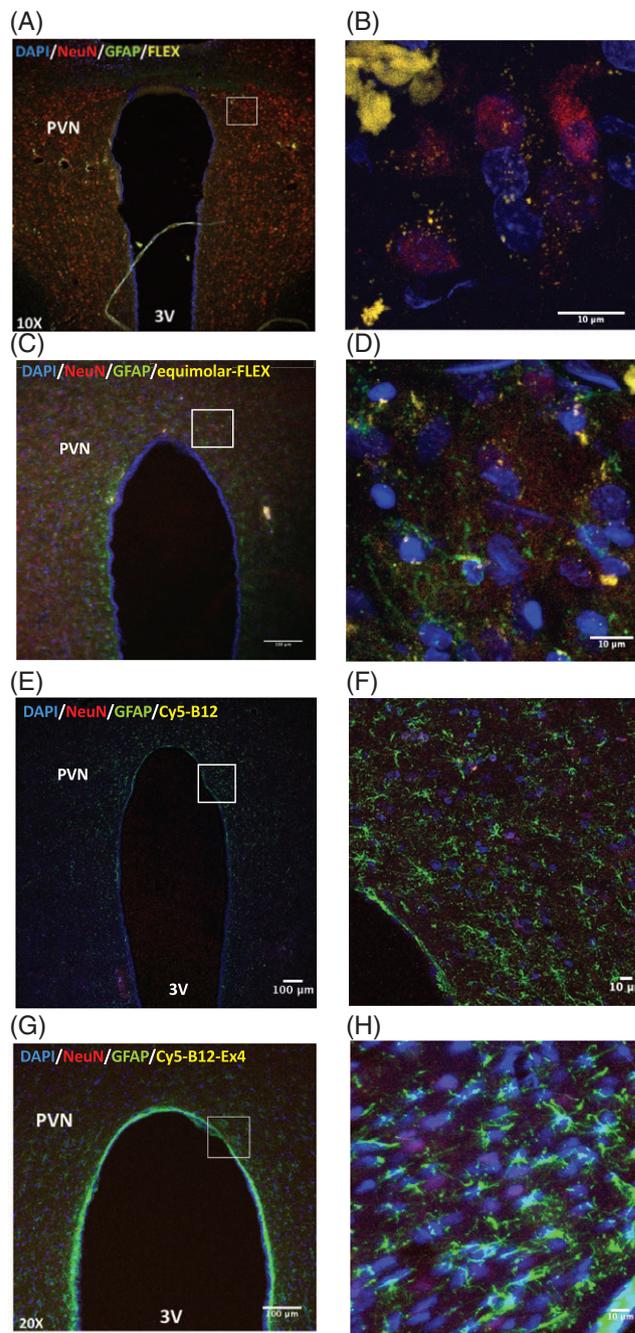
FIGURE 6 Systemically-delivered fluorescently labeled Ex-4 (Flex) highly penetrates within the PVN, whereas Cy5-B12 and Cy5-B12-Ex4 do not. A, C, E, G, Images were acquired at 10-20× or B, D, F, H, 63× (with 2-3× optical zoom) magnifications. Brains were processed for immunohistochemistry to label Flex, Equimolar-Flex, Cy5-B12 and Cy5-B12-Ex4 (yellow), astrocytes (GFAP) (green) and neurons (NeuN) (red). Sections were counterstained using DAPI (blue) to visualize cell nuclei. B, Flex and D, equimolar-Flex immunoreactivity is readily visualized in neurons in the PVN. F, Cy5-B12 and H, Cy5-B12-Ex4 are not present either in neurons or in astrocytes within the PVN. Abbreviations: 3V, third ventricle; PVN, paraventricular hypothalamic nucleus

4 | DISCUSSION

GLP-1-based pharmacotherapies for T2DM have been revolutionary in providing largely safe and efficacious means to reduce chronic hyperglycaemia (see References 1,3,15 for review). However, because of the side effects of current GLP-1-based compounds, including anorexia, nausea and vomiting, nearly 1 in 4 T2DM patients are not able to benefit from the full

(A)

80 um

(B)

10 um
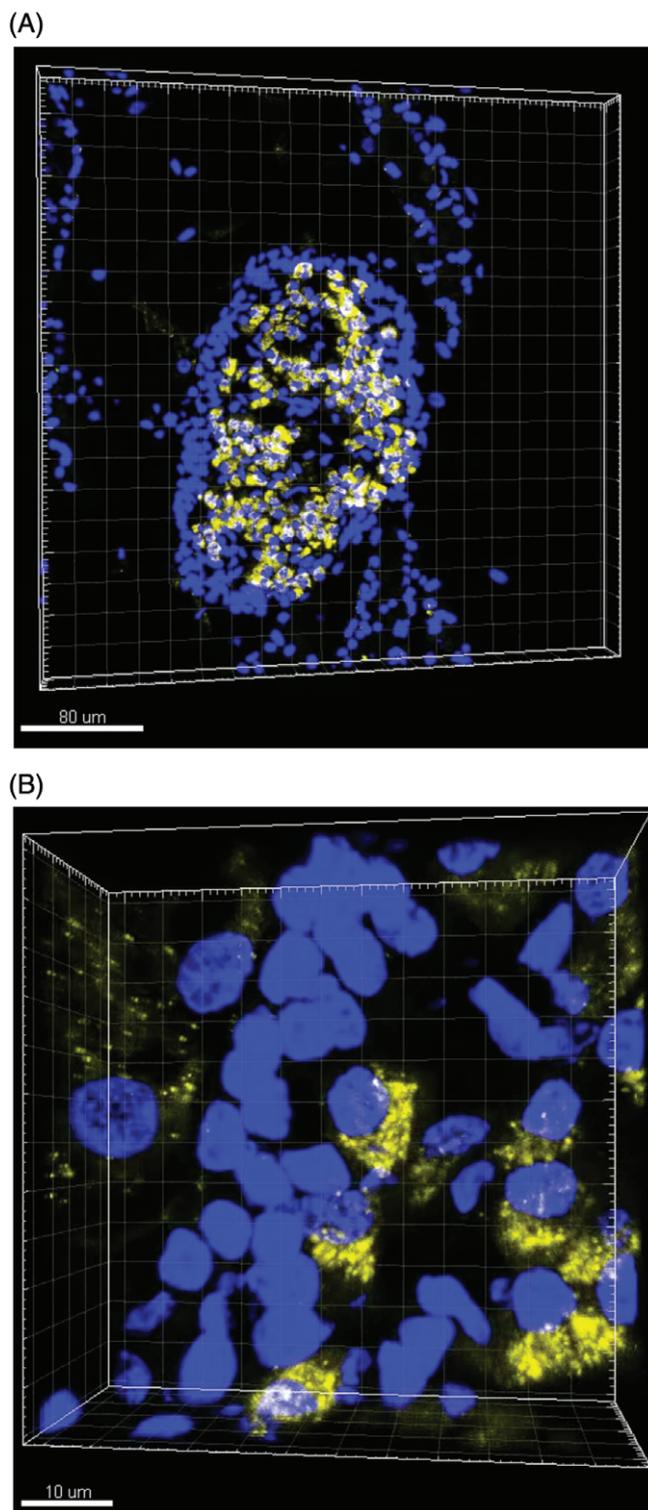
FIGURE 7 Systemically-delivered Cy5-B12-Ex4 is colocalized with insulin in the pancreas. The representative still images from 3-dimensional rotational videos (Videos S1 and S2) demonstrate that Cy5-B12-Ex4 (yellow) is colocalized with insulin (red) in pancreatic beta cells. Sections were counterstained with DAPI (blue). A, Videos and corresponding representative images were taken from a z-stack (2 μm step size) at 20× (Video S1) and B, from a z-stack (1μm step size) at 40× with 4-5× optical zoom (Video S2)

pharmaceutical advantages of these pharmacotherapies.[10–14] There is clearly a critical need to develop a new generation of GLP-1 pharmacotherapies that provide hypoglycaemic benefit without

eliciting detrimental side effects. Although the hypophagic effects of GLP-1R agonists are often attractive to clinicians and to T2DM patients with overweight or obesity, much of the same CNS circuitry underlying GLP-1R ligand-mediated anorexia is also partially responsible for mediating nausea/malaise.[8] Moreover, weight loss may be undesirable for some T2DM patients, such as individuals with a normal BMI. As the hypophagia and illness-like effects of existing GLP-1R agonists require CNS penetrance and direct central action,[8,21–23] we sought to create a GLP-1R agonist conjugate that minimizes anorexia and nausea by reducing CNS penetrance, but that retains potent pharmacodynamics and a pharmacokinetic profile for peripheral GLP-1R populations to exert glycaemic benefits. This report shows for the first time (see Reference 30 for previous biochemical GLP-1R agonism analyses) the ability of B12-Ex4 to improve glucose tolerance in rodents without producing hypophagia, body weight loss or CTA. Supporting a recent finding showing that B12 is not substantially transported into the adult mouse brain,[40] current immunohistochemical data suggest that the unique profile of glycaemic effects without the same hypophagic/CTA-producing effects of Ex4 involves a direct effect of B12-Ex4 on pancreatic beta cells, coupled with a virtual absence of CNS penetrance of the compound.

The rat provides a unique model for the proof-of-concept testing needed for preclinical evaluation of B12-Ex4. Rats show an unexpected hyperglycaemic response to Ex4, explained, in part, by a CNS-mediated activation of the sympathetic nervous system.[41] In addition, as humans, rats show a pronounced profile of behavioral effects to systemic Ex4, including reduced food intake and body weight, as well as illness-like behaviors, again explained by CNS action.[8,22,64] B12-Ex4 did not produce the same suppression of food intake, reduction in body weight and induction of CTA as did Ex4 in rats. The effect of B12-Ex4 on glycaemic control was also evaluated in mice, a species in which Ex4 produces a hypoglycaemic response similar to that observed in humans. In mice, B12-Ex4 and unconjugated Ex4 each elicited hypoglycaemic responses in an IPGTT. Collectively, these data provide an ideal preclinical set of outcomes to support the therapeutic potential of this conjugate as a future antidiabetic drug for humans.

The in vivo behavioral data were supported by our immunohistochemical analyses, showing a virtual absence of B12-Ex4 CNS penetrance in the DVC and PVN, 2 areas of the brain showing unconjugated Ex4 penetrance, and believed to mediate, in part, the hyperglycaemic, hypophagic, body weight-suppressive and malaise-producing effects of Ex4 in rats. Future studies are warranted to identify the mechanisms responsible for the minimal CNS uptake of B12 and the molecular mechanisms by which B12 conjugation reduces CNS Ex4 access. It will also be important to address whether higher doses of B12-Ex4 are able to more effectively penetrate the CNS. The 5 μg/kg dose of Cy5-B12-Ex4 used for this study was selected because 5 μg/kg B12-Ex4 had no effect on feeding or body weight in rats, but produced hypoglycaemia in the OGTT, suggesting that a lower dose of B12-Ex4 elicits an optimal profile of glycaemic and energy balance effects. In contrast, a higher dose of B12-Ex4 (20 μg/kg) reduced blood glucose but also caused a small but significant transient suppression of feeding, suggesting

that higher doses may have a slightly different pattern of effects. Nevertheless, these results clearly underscore the reduced CNS penetrance, but retention of glycaemic benefits, with lower doses of B12-Ex4.

As B12-Ex4 does not extensively penetrate into the CNS, pancreatic GLP-1R represents the probable cellular substrate mediating the hypoglycaemic effects of B12-Ex4. Further analyses supported this hypothesis, as immunohistochemical data showed colocalization of Cy5-B12-Ex4 with insulin in the pancreas. This suggests that B12-Ex4 may exert its glycaemic effects via direct action at pancreatic beta cells, while CNS-mediated effects of GLP-1R activation such as hypophagia, nausea and malaise are minimal or absent because of the absence of penetrance of B12-Ex4 into the brain, consistent with previously reported radio-probe data.[40]

The current data provide novel mechanistic evidence that B12 conjugation to a GLP-1R agonist can be used as a means to retain the hypoglycaemic properties of GLP-1R agonists while greatly reducing the CNS-mediated anorexia and illness effects observed with currently approved GLP-1-based ligands.[3,8,9] These studies are far from the complete set of in vivo glycaemic analyses needed for B12-Ex4, but certainly justify the need for more comprehensive future analyses. Further investigations are warranted to examine the acute actions of B12-Ex4 in diabetic animal models, as well as to evaluate the metabolic effects of chronic B12-Ex4 administration. It will also be critical to evaluate whether, and to what extent, B12-Ex4 may localize within other CNS nuclei not examined here. Collectively, these data highlight the discovery that B12 conjugation to Ex4 results in a next-generation incretin therapeutic with the clinically desired hypoglycaemic effects, but without concomitant hypophagia, body weight loss and, most notably, illness-like behaviors, which is ideal for the future of T2DM treatment in humans. This method of conjugation may also be broadly beneficial to other therapeutics that would benefit from reduced CNS penetrance.

Conflict of interest

M. R. H. and E. G. M-B. receive funding from Zealand Pharma that was not used in support of these studies. M. R. H. receives funding from Novo Nordisk that was not used in support of these studies. R. P. D. is a scientific advisory board member and receives funding from Xeragenx LLc, St. Louis, Missouri that was used, in part, to support these studies. R. P. D. is a scientific advisory board member of Ichor Therapeutics, Lafayette, New York and of Balchem, New Hampton, New York and receives funds from both that were not used in support of these studies. R. P. D. is the named author of a patent pursuant to this work that is owned by Syracuse University. The authors declare no other competing financial interests or conflicts of interest.

Author contributions

R. P. D. and M. R. H. conceived of the project. R. P. D. and M. R. H. conceptualized the hypotheses and designed the experiments. J. L. W., R. L. B. and R. P. D. designed and synthesized the compounds. E. G. M-B., C. G. L., J. L. W., R. L. B., T. B., D. J. R., K. K-L., L. E. M., R. L. and L. M. S. conducted experiments and collected data. E. G. M.-B, C. G. L., T. B., D. J. R., L. M. S., B. C. D. J., G. G. H., R. P. D., and M. R. H. analyzed data. E. G. M-B., C. G. L., T. B., B. C. D. J., R. P. D. and M. R. H. wrote the manuscript. All authors edited the manuscript. R. P. D. and M. R. H. are guarantors for this work.

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