Diabetes Publish Ahead of Print, published online September 9, 2009

Differences in the central anorectic effects of GLP-1 and exendin-4 in rats

Running title: Central GLP-1 and exendin-4

Jason G. Barrera[1], David A. D'Alessio[2], Daniel J. Drucker[3], Stephen C. Woods[1], and Randy J. Seeley[1]

[1]Department of Psychiatry, University of Cincinnati, Cincinnati, OH, 45237
[2]Department of Internal Medicine, University of Cincinnati, Cincinnati, OH, 45237
[3]Department of Medicine, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, University of Toronto, Toronto, ON, M5T 3L5, Canada

To whom correspondence should be addressed:
Jason G. Barrera
E-Mail: barrerjg@email.uc.edu

Submitted 24 February 2009 and accepted 26 August 2009.

Objective: Glucagon-like peptide-1 (GLP-1) is a regulatory peptide synthesized in the gut and the brain that plays an important role in the regulation of food intake. Both GLP-1 and exendin-4 (Ex4), a long-acting GLP-1 receptor (GLP-1r) agonist, reduce food intake when administered intracerebroventricularly (i3vt), whereas Ex4 is much more potent at suppressing food intake when given peripherally. It has generally been hypothesized that this difference is due to the relative pharmacokinetic profiles of GLP-1 and Ex4, but it is possible that the two peptides control feeding via distinct mechanisms.

Research Design and Methods: In this study, the anorectic effects of i3vt GLP-1 and Ex4, and the sensitivity of these effects to GLP-1r antagonism, were compared in rats. In addition, the GLP-1r-dependence of the anorectic effect of i3vt Ex4 was assessed in GLP-1r-/- mice.

Results: I3vt Ex4 was 100-fold more potent than GLP-1 at reducing food intake, and this effect was insensitive to GLP-1r antagonism. However, GLP-1r antagonists completely blocked the anorectic effect of intraperitoneal Ex4. Despite the insensitivity of i3vt Ex4 to GLP-1r antagonism, i3vt Ex4 failed to reduce food intake in GLP-1r-/- mice.

Conclusions: These data suggest that, although GLP-1r are required for the actions of Ex4, there appear to be key differences in how GLP-1 and Ex4 interact with CNS GLP-1r, and in how Ex4 interacts with GLP-1r in the brain versus the periphery. A better understanding of these unique differences may lead to expansion and/or improvement of GLP-1-based therapies for type 2 diabetes and obesity.

Glucagon-like peptide-1 (GLP-1) is a product of the preproglucagon gene (1) that is synthesized in the distal ileum (2) as well as the caudal nucleus of the solitary tract (NTS) and ventrolateral medulla (3). Although GLP-1 is perhaps best known for its essential role in the regulation of peripheral glucose homeostasis, multiple lines of evidence suggest that GLP-1 also acts in the central nervous system (CNS) to regulate food intake. In support of this hypothesis, GLP-1 receptors (GLP-1r) are expressed in brain regions known to regulate energy balance, such as the mediobasal hypothalamus and the caudal brainstem (3; 4), and consistent with a role for GLP-1 as a putative satiety signal, central administration of GLP-1 potently reduces short-term food intake (5; 6). Conversely, central administration of the GLP-1r antagonist exendin (9-39) (Ex9) increases food intake and body weight (7), suggesting that endogenous GLP-1 has a physiological role in the regulation of energy balance.

Recently, the GLP-1 system has emerged as a novel therapeutic target for type 2 diabetes, as peripheral GLP-1 infusion effectively lowers blood glucose levels and improves glucose tolerance in humans (8). However, because circulating active GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-4 (DPP-4) (9-11), alternative strategies for targeting the GLP-1 system have been developed, including stable GLP-1 analogues and DPP-4 inhibitors. One such analogue is exendin-4 (Ex4), a peptide originally isolated from the saliva of the Gila monster (*Heloderma suspectum*), which is a highly potent, DPP-4 resistant GLP-1r agonist *in vitro* and *in vivo* (12; 13). Recently, exenatide (a synthetic Ex4) and the DPP-4 inhibitor sitagliptin were FDA-approved as therapies for type 2 diabetes. However, whereas both drugs effectively improved glycemic control in clinical trials (14; 15), Ex4, but not sitagliptin, was also associated with significant weight loss (14; 16).

The above finding is compelling in that it raises the possibility that Ex4, at doses used clinically, may have *in vivo* actions that are substantively different from those of intact GLP-1 achieved through DPP-4 inhibition. Although studies using GLP-1r knockout (GLP-1r-/-) mice provide strong evidence that the GLP-1r is necessary for the *in vivo* actions of Ex4 (17-21), other studies using GLP-1r antagonists suggest that Ex4, particularly in the brain, may act at least in part independently of GLP-1r (22-24). Therefore, we tested the hypothesis that the central anorectic effect of Ex4 is different from that of GLP-1.

MATERIALS AND METHODS

Animals: Adult male Long-Evans rats (Harlan, Indianapolis, IN), GLP-1r-/- mice and their wild-type (WT) C57BL/6J littermates were housed individually in plastic rodent cages and maintained on a 12-hour light/dark cycle with *ad libitum* access to water and pelleted rodent chow (Harlan Teklad). Rats and mice were outfitted with cannulas (Plastics One, Roanoake, VA) aimed at the 3rd-cerebral ventricle, and correct cannula placement was verified as previously described (25; 26). All procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee (IACUC).

***Peptides*:** GLP-1 and Ex4 were obtained from Bio Nebraska (Lincoln, NE) and American Peptide (Sunnyvale, CA), respectively. The GLP-1r antagonists des His1, Glu8 exendin-4 (dHEx) and exendin (9-39) (Ex9) were obtained from Baylor College of Medicine Protein Synthesis Core (Houston, TX) and Tocris (Ellisville, MO), respectively. All peptides were dissolved in saline and administered either intracerebroventricularly (i3vt) in a volume of 1.0 μl or

intraperitoneally (ip) in a volume of 1.0 ml/kg.

Food Intake Studies: Rats and mice were fed *ad libitum* at all times except for the mornings of study days. During this time, food was removed from the animals' cages and weighed 4 h before lights off, and animals were assigned to weight-matched groups. Pretreatment (saline or GLP-1r antagonist) injections commenced 1 h before lights off, and treatment (saline or GLP-1r agonist) injections commenced 30 min before lights off. Injection order was counterbalanced across all experimental groups to evenly distribute subtle variations in timing of injections. Food was returned to the animals' cages at lights off, and food intake and body weight were measured at selected time points.

First, dose-response curves for anorexia induced by i3vt GLP-1 (0, 0.3, 1.0, 3.0 and 10.0 μg) and Ex4 (0, 0.01, 0.03, 0.1 and 0.3 μg) were established. Based on these results, time courses of anorexia induced by i3vt GLP-1 (3.0 nmol, ~10.0 μg) and Ex4 (0.03 nmol, ~0.1 μg) were compared, and conditioned taste aversion (CTA) to the same doses of i3vt GLP-1 and Ex4 was assessed as previously described (27).

To assess the ability of central GLP-1r antagonism to block anorexia induced by central GLP-1 and Ex4, rats were pretreated with i3vt saline, dHEx (10.0 μg, (28)), or Ex9 (100.0 μg) and then treated with i3vt saline, GLP-1 (10.0 μg), or Ex4 (0.1 μg). To assess the ability of peripheral GLP-1r antagonism to block anorexia induced by peripheral Ex4, rats were pretreated with ip saline or dHEx (1.0 mg/kg) and then treated with ip saline or Ex4 (10.0 μg/kg). Finally, to assess the ability of central Ex4 to reduce food intake in GLP-1r-/- mice, mice were treated with i3vt saline or Ex4 (1.0 μg).

c-Fos Immunohistochemistry: To assess the ability of central GLP-1r antagonism to block neuronal activation induced by central GLP-1 and Ex4, rats were pretreated with i3vt saline or dHEx (10.0 μg) and then treated with i3vt saline, GLP-1 (10.0 μg) or Ex4 (0.1 μg). Two hours later, rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS) followed by 4.0% paraformaldehyde/PBS. Brains were postfixed at 4°C for 24 h in 4.0% paraformaldehyde/PBS and stored at 4°C in 30.0% sucrose/PBS. Serial coronal forebrain sections and longitudinal hindbrain sections were collected at 35 μm using a freezing microtome and stored at -20°C in cryoprotectant.

After washing with PBS, sections were incubated in 1.0% hydrogen peroxide/PBS for 10 min followed by 1.0% sodium borohydride/PBS for 30 min. Sections were blocked for 1 h in 0.1% BSA/0.4% Triton-X-100/PBS and incubated overnight at room temperature in blocking solution containing rabbit anti-c-Fos diluted at 1:5,000 (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA). The next morning, sections were washed and incubated at room temperature for 1 h in blocking solution containing biotinylated goat anti-rabbit IgG diluted at 1:200 (BA-1000, Vector Laboratories, Burlingame, CA) followed by 1 h in ABC solution diluted 1:800 in PBS (PK6100, Vector Laboratories, Burlingame, CA) and 10 min in DAB-nickel solution. Finally, sections were washed with 0.1 M phosphate buffer (PB), mounted on gelatin-coated slides, and coverslipped.

For quantification of c-Fos immunoreactivity (c-Fos IR) in the central nucleus of the amygdala (CeA), paraventricular nucleus of the hypothalamus (PVN) and nucleus of the solitary tract (NTS), digital images of sections were acquired using a digital camera attached to a Zeiss microscope (Zeiss, Thornwood, NY). For each brain, two sections per area were analyzed, and special care was taken to compare only sections within the same plane

along the rostro-caudal (CeA and PVN) or dorso-ventral (NTS) axis. c-Fos IR was quantified as optical density using the NIH program Scion Image.

Tissue Culture Studies: INS-1 cells were seeded in 35-mm 6-well plates at a density of 2 x 10^5 cell/well in 1.5 ml of media consisting of RPMI-1640 supplemented with 10% heat-inactivated FBS, 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 50.0 μM β-mercaptoethanol and 0.5 mg/ml gentamicin sulfate and grown in a 37°C incubator in an atmosphere of 5% CO_2 and 95% air and 100% humidity for 3 days until nearly confluent. On Day 4, cells were washed with PBS and replaced with fresh media. On Day 5, cells were pre-incubated for 2 h in 2.0 ml of buffer consisting of KRB supplemented with 0.1% BSA and 30 mg/dl glucose and then washed twice with 2.0 ml of the same buffer solution. Cells were then incubated for 1 h in 1.0 ml of KRB supplemented with 0.1% BSA, 200 mg/dl glucose and either 1.0 nM GLP-1, 0.01 nM Ex4 or 1.0 nM Ex4 with or without 100 nM dHEx. Finally, incubation buffer was harvested, centrifuged, decanted and stored at -20°C for IRI assay, and cells were washed once with 1.0 ml of pre-incubation buffer and then extracted with 1.0 ml of acid ethanol for 2 h at -20°C, after which acid ethanol was diluted 1:200 with Tris assay buffer for IRI assay in cell layer. IRI was measured using a radio-immunoassay as previously described (29).

Statistical Analysis: All values are reported as mean ± SEM. Data were analyzed using one- or two-way ANOVA or two-way repeated-measures ANOVA. Post-hoc multiple comparisons were made using Tukey's post-hoc test. Significance was set at $p<0.05$ for all analyses.

RESULTS

Comparison of i3vt GLP-1 and Ex4-induced anorexia. Consistent with previous reports, i3vt GLP-1 and Ex4 elicited potent, dose-dependent reductions in 4-h food intake (Fig. 1A and B; $p<0.05$, one-way ANOVA with Tukey's post-hoc test). However, Ex4 significantly reduced food intake at doses much lower than those of GLP-1. Specifically, 10.0 μg of GLP-1 and 0.1 μg of Ex4 produced comparable degrees of anorexia, reducing food intake to 56 and 45% of control values, respectively. These data indicate that, when administered into the 3rd ventricle, Ex4 is roughly 100-fold more potent than GLP-1 at reducing food intake.

Figure 1C illustrates the time course of i3vt GLP-1- and Ex4-induced anorexia. Whereas 3.0 nmol (~10.0 μg) of GLP-1 and 0.03 nmol (~0.1 μg) of Ex4 both actively suppressed food intake up to 4 h, only Ex4 elicited persistent anorexia that remained detectable throughout the 24 h of observation ($p<0.05$, two-way repeated-measures ANOVA with Tukey's post-hoc test). Furthermore, these doses of GLP-1 and Ex4 both led to the formation of a CTA (Fig. 1D; $p<0.05$, one-way ANOVA with Tukey's post-hoc test). Interestingly, there was a strong trend toward a significantly lower preference ratio of Ex4-treated rats versus GLP-1-treated rats ($p=0.052$), suggesting that the aversive effects of Ex4 were more pronounced than those of GLP-1.

Sensitivity of i3vt GLP-1 and Ex4 to GLP-1r antagonism. Although previous studies have reported an inability to block certain effects of Ex4 with GLP-1r antagonists, these studies did not necessarily account for the significantly greater potency of Ex4 over GLP-1. Therefore, we sought to compare the ability of GLP-1r antagonists to block anorexia and neuronal activation induced by doses of i3vt GLP-1 and Ex4 that produce effects of comparable magnitude. Pretreatment with either 10.0 μg of dHEx or 100.0 μg of Ex9 caused near-complete blockade of anorexia induced by 10.0 μg of GLP-1 (Fig. 2A and C; $p<0.05$ by two-way ANOVA with Tukey's post-hoc test).

However, whereas 0.1 μg of Ex4 and 10.0 μg of GLP-1 elicited comparable degrees of anorexia, the doses of dHEx and Ex9 that nearly abolished GLP-1-induced anorexia failed to block the anorectic effect of Ex4 (Fig. 2B and D), although a non-significant trend was observed with dHEx (P=0.148).

To determine whether neuronal activation in response to GLP-1 and Ex4 was also differentially sensitive to GLP-1r antagonism, the effect of i3vt dHEx to block c-Fos immunoreactivity (IR) induced by i3vt GLP-1 and Ex4 was compared. At the same doses as used above, GLP-1 and Ex4 both induced c-Fos IR in identical brain regions, including the CeA, PVN and the NTS (Fig. 3A, B, and C; $p<0.05$ by two-way ANOVA with Tukey's post-hoc test). The magnitude of c-Fos IR induced by GLP-1 and Ex4 was similar in the PVN and NTS, whereas GLP-1 induced slightly more c-Fos IR than Ex4 in the CeA. In the CeA, dHEx significantly blocked c-Fos IR induced by GLP-1 ($p<0.05$); however, in the PVN and the NTS, this difference failed to reach statistical significance. Nonetheless, for all three regions, the amount of c-Fos IR in brains treated with dHEx and Ex4 was significantly greater than that of brains treated with saline, dHEx alone, or dHEx and GLP-1 ($p<0.05$). These results, combined with the food intake data, suggest that CNS actions of Ex4 are relatively insensitive to competitive GLP-1r antagonism.

Potency of Ex4 and sensitivity to GLP-1r antagonism *in vitro.* Because dHEx, a validated but lesser used GLP-1r antagonist (28; 30; 31), failed to block anorexia and neuronal activation induced by i3vt Ex4, we sought to determine whether dHEx is an effective antagonist of Ex4 *in vitro* by assessing its ability to block insulin secretion induced by Ex4 in the rat pancreatic islet cell line INS-1. As expected, 1.0 nM GLP-1 significantly augmented insulin secretion above that of glucose alone, and this effect was completely blocked by co-incubation with 100 nM dHEx (Fig. 4; $p<0.05$ by two-way ANOVA with Tukey's post-hoc test). However, in contrast to our *in vivo* data, 0.01 nM Ex4 failed to augment insulin secretion, whereas 1.0 nM Ex4 had an effect that was comparable to 1.0 nM GLP-1. Moreover, this effect was completely blocked by co-incubation with 100 nM dHEx ($p<0.05$).

Sensitivity of ip Ex4 to GLP-1r antagonism. To determine whether the insensitivity of Ex4 to GLP-1r antagonism was specific to CNS administration, we assessed the ability of dHEx to block anorexia induced by ip Ex4. As expected, 10 μg/kg of ip Ex4 significantly reduced food intake at 4 h (Fig. 5; $p<0.05$ by two-way ANOVA with Tukey's post-hoc test). Surprisingly, pretreatment with 1.0 mg/kg of ip dHEx, the same 100-fold excess of antagonist that failed to block anorexia induced by i3vt Ex4, significantly attenuated this effect ($p<0.05$).

Effect of i3vt Ex4 in WT and GLP-1r-/- mice. The insensitivity of CNS Ex4 effects to GLP-1r antagonism raises the possibility that Ex4 may act in part via a GLP-1r-independent mechanism. To determine whether the GLP-1r is required for the central anorectic effect of Ex4, i3vt Ex4 was administered to WT and GLP-1r-/- mice. In WT mice, 1.0 μg of i3vt Ex4 elicited profound anorexia such that daily food intake and body weight were significantly reduced for up to 48 and 72 h, respectively (Fig. 6A and B; $p<0.05$ by two-way repeated-measures ANOVA with Tukey's post-hoc test). Conversely, this same high dose of i3vt Ex4 had no effect on food intake or body weight in GLP-1r-/- mice (Fig. 6C and D).

DISCUSSION

Because Ex4 (14), but not the DPP-4 inhibitor sitagliptin (16), produces weight loss in patients, it is critical that we better understand the unique anorectic properties of Ex4. To this end, we report key distinctions

between the central anorectic effects of Ex4 and native GLP-1. Not only do our data confirm that central GLP-1 and Ex4 differ significantly in potency and duration of action, but they also reveal novel differences between the two peptides regarding sensitivity to GLP-1r antagonism.

Ex4, when administered into the CNS, reduces food intake in a manner distinct from that of GLP-1. Consistent with previous reports (32; 33), central Ex4 reduced 4-h food intake at doses 30- to 100-fold lower than those required by GLP-1 to cause equivalent anorexia. Importantly, this difference in potency at 4 h cannot simply be explained by differences in duration of action, as both 3.0 nmol of GLP-1 and 0.03 nmol of Ex4 reduced food intake to a comparable extent from 0-2 and 2-4 h. However, in contrast to GLP-1, Ex4 dynamically reduced food intake over 24 h of observation, indicating that, even at significantly lower doses, central Ex4 exhibits a significantly longer duration of action.

Consistent with our food intake data, 0.1 μg of central Ex4 produced an almost identical degree of neuronal activation as 10.0 μg of GLP-1 in the PVN and the NTS, but interestingly not the CeA. It is possible that, because 100-fold less Ex4 than GLP-1 was administered, less peptide diffused through the neuropil to the CeA, which, unlike the PVN and the NTS, does not abut the ventricular system. Despite producing less neuronal activation in the CeA, an area important for the formation of GLP-1-mediated CTA (34), central Ex4 produced a lower preference ratio for saccharin than GLP-1, suggesting that Ex4 induced a greater visceral illness response. Although a proportional relationship between GLP-1r-mediated neuronal activity and behavioral responses has yet to be established, these data are significant because they suggest that enhanced visceral illness or aversive learning may in part underlie the potent anorectic effect of central Ex4.

The above data and those of others support a role for Ex4 as a highly potent, long-acting CNS GLP-1r agonist, yet the mechanism for this unique pharmacological profile remains unknown. One possibility is that GLP-1 and Ex4 bind differently to CNS GLP-1r. However, *in vitro* and *ex vivo* comparisons of binding affinity have yielded equivocal results (35-38), and it remains unclear whether GLP-1 and Ex4 remain bound to CNS GLP-1r for different periods of time. A second possibility is that GLP-1 and Ex4 differentially desensitize CNS GLP-1r. However, data from Baggio and colleagues revealed no difference in the ability of GLP-1 and Ex4 to desensitize the GLP-1r *in vitro* (39). Moreover, because GLP-1 and Ex4 were administered as boluses, this hypothesis fails to adequately explain the present results. Finally, it is possible that differential clearance and/or degradation of GLP-1 versus Ex4 account for their distinct pharmacological profiles within the CNS.

Perhaps the most striking difference between central GLP-1 and Ex4 revealed by our data is their sensitivity to GLP-1r antagonism. Whereas dHEx almost completely blocked anorexia and neuronal activation induced by GLP-1, it failed to significantly block that induced by an equipotent dose of Ex4. This phenomenon is not specific to dHEx, as Ex9 also failed to block anorexia induced by central Ex4. However, dHEx is an effective antagonist of Ex4 *in vitro*, as it completely blocked the enhancement of glucose-stimulated insulin secretion induced by Ex4 in INS-1 cells. In addition, dHEx is an effective antagonist of Ex4 *in vivo*, as ip dHEx completely blocked anorexia induced by ip Ex4. Taken together, these data indicate that, compared to GLP-1, Ex4 is relatively insensitive to GLP-1r antagonism. Moreover, this phenomenon seems to be specific for central effects but not peripheral effects, many of which have been reported to be blocked by Ex9 (40-42).

Certainly, previous studies have reported an inability to block Ex4 effects with GLP-1r antagonists (22-24). However, they may not have adequately accounted for the increased potency of Ex4 versus GLP-1. Here, we closely controlled for this difference and found that pretreatment with GLP-1r antagonists significantly blocked anorexia induced by central GLP-1 but not an equipotent and, importantly, 100-fold lower dose of Ex4. Moreover, this phenomenon is not secondary to differences in agonist duration of action, as it was observed at early time points when both GLP-1 and Ex4 dynamically reduced food intake. Nor is it secondary to the antagonist duration of action, as timing of pretreatment and treatment injections was consistent across all experiments, and similar trends were observed with both c-Fos and food intake (data not shown) at 2 h.

While intriguing, these data are difficult to reconcile with our other experiments. Specifically, we found no differences between GLP-1 and Ex4 in either potency or sensitivity to dHEx *in vitro*, although this discrepancy might easily be explained by obvious differences between animal models and immortalized cell lines. More difficult to explain, however, is the comparison to our peripheral Ex4 food intake study, in which the same 100-fold excess of dHEx, this time administered ip, completely blocked ip Ex4-induced anorexia. Consequently, it is possible that fundamental differences exist between central and peripheral GLP-1r, which may occur at the level of post-translational processing, protein-protein interactions, or coupling to second-messenger systems.

Perhaps the most obvious explanation for the discrepancies between central GLP-1 and Ex4 is that the latter acts in part independently of the GLP-1r. However, consistent with previous reports (18), central Ex4 had no effect on either food intake or body weight in GLP-1r-/- mice, suggesting that the GLP-1r is required for these effects. Although the lack of Ex4 effects in GLP-1r-/- mice provides a strong basis to rule out GLP-1r-independence, there is some evidence for both functional (43) and structural (44) differences between the GLP-1 systems of mice and rats. Recently, Sowden and colleagues reported that Ex4 increases heart rate in rats but not mice (20). Finally, other studies reporting an inability to block Ex4 effects with GLP-1r antagonists have all been conducted in rats (22-24). Although none of these observations provides definitive evidence for GLP-1r-independent effects of Ex4, they do raise the possibility that Ex4 may interact with the GLP-1r in a species-dependent manner.

Although difficult to reconcile with the above data, our findings regarding central Ex4 and GLP-1r antagonists are consistent with several reports of *in vivo* effects of Ex4 that are insensitive to GLP-1r antagonists (22-24). Recently, it was reported that central Ex4 decreases ghrelin secretion in fasted rats (24). Not only was this effect insensitive to Ex9 blockade, it was also elicited by Ex9 alone, consistent with several *in vitro* reports of independent Ex9 effects (45-50). Whereas these data, like ours, fail to prove GLP-1r-independence of Ex4, they are nonetheless significant in that they provide potential mechanistic insight into the unique anorectic properties of central Ex4, particularly its duration of action. For instance, ongoing GLP-1r signaling by Ex4 may prevent circulating ghrelin levels from rising in response to Ex4-mediated reductions in food intake, leading to an attenuation or delay in the subsequent drive to eat and thus a prolonged duration of anorexia. However, because our experiments used *ad libitum* fed rats, whose circulating ghrelin levels should be low, and because Ex4 is more efficacious in fed versus fasted rats (51), it seems unlikely that Ex4's effects on ghrelin

secretion underlie either its increased potency acutely or its insensitivity to GLP-1r antagonists in the present studies.

Because studies have generally found no effect of Ex4 in GLP-1r-/- mice (17-21), it seems reasonable to cite strictly pharmacological differences when explaining discrepancies between *in vivo* effects of GLP-1 and Ex4. However, in many ways the existing data fail to adequately support this hypothesis. For instance, some *in vitro* studies have found Ex4 to have greater potency and affinity for the GLP-1r than native GLP-1 (35), but these differences, at least in potency, are significantly smaller than those reported here. Regarding antagonist sensitivity, one potential explanation for our findings is that Ex4 is more able to displace antagonists from the GLP-1r. However, studies have generally reported little-to-no difference in the ability of GLP-1 versus Ex4 to displace radio-labeled Ex9 (52-55). Taken together, our data, combined with the existing literature, provide conclusive evidence for distinct pharmacological profiles of GLP-1 and Ex4, yet further studies are needed to understand whether pharmacological differences alone are sufficient explain the unique *in vivo* effects of Ex4.

In conclusion, our data indicate that the central, but not peripheral, anorectic effect of Ex4 is insensitive to GLP-1r antagonism, yet the GLP-1r is required for this effect. These data suggest that there are important differences between the *in vivo* pharmacological properties of GLP-1 and Ex4 within the CNS. Moreover, they underscore the need for a greater understanding of how these ligands interact with CNS GLP-1r, particularly in light of recent data revealing novel roles of CNS GLP-1r activity in the regulation of peripheral glucose homeostasis and cardiovascular function (56; 57). Such an understanding is critical if we are to maximize the therapeutic benefit of Ex4 and other GLP-1-based therapies.

ACKNOWLEDGEMENTS

We thank Kathleen Smith, Joyce Sorrell and Jeanette Teague for their expert technical assistance. This work was supported by National Institutes of Health (NIH) RO1 DK54890 to R.J.S., American Diabetes Association Physician Scientist Training Award 7-06-PST-02 to J.G.B., NIH RO1 DK57900 to D.A.D., JDRF 1-2006-796 to D.J.D. and research funding from Amylin Pharmaceuticals.

REFERENCES

1. Lopez LC, Frazier ML, Su CJ, Kumar A, Saunders GF: Mammalian pancreatic preproglucagon contains three glucagon-related peptides. *Proc Natl Acad Sci USA* 80:5485-5489, 1983

2. Varndell IM, Bishop AE, Sikri KL, Uttenthal LO, Bloom SR, Polak JM: Localization of glucagon-like peptide (GLP) immunoreactants in human gut and pancreas using light and electron microscopic immunocytochemistry. *J Histochem Cytochem* 33:1080-1086, 1985

3. Merchenthaler I, Lane M, Shughrue P: Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. *J Comp Neurol* 403:261-280, 1999

4. Shimizu I, Hirota M, Ohboshi C, Shima K: Identification and localization of glucagon-like peptide-1 and its receptor in the brain. *Endocrinology* 121:1076-1082, 1987

5. Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR: A role for glucagon-like peptide-1 in the central regulation of feeding [see comments]. *Nature* 379:69-72, 1996

6. Tang-Christensen M, Larsen PJ, Goke R, Fink-Jensen A, Jessop DS, Moller M, Sheikh SP: Central administration of GLP-1-(7--36) amide inhibits food and water intake in rats. *American Journal of Physiology* 271:R848-856, 1996

7. Meeran K, O'Shea D, Edwards CM, Turton MD, Heath MM, Gunn I, Abusnana S, Rossi M, Small CJ, Goldstone AP, Taylor GM, Sunter D, Steere J, Choi SJ, Ghatei MA, Bloom SR: Repeated intracerebroventricular administration of glucagon-like peptide-1-(7-36) amide or exendin-(9-39) alters body weight in the rat. *Endocrinology* 140:244-250, 1999

8. Kreymann B, Ghatei MA, Williams G, Bloom SR: Glucagon-like peptide-1 7-36: A physiological incretin in man. *Lancet* 2:1300-1303, 1987

9. Mentlein R, Gallwitz B, Schmidt WE: Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1 (7-36) amide, petide histidine methionine and is responsible for their degradation in human serum. *Eur J Biochem* 214:829-835, 1993

10. Deacon CF, Nauck MA, Toft-Nielsen M, Pridal L, Willms B, Holst JJ: Both subcutaneously and intravenously administered glucagon-like peptide I are rapidly degraded from the NH2-terminus in type II diabetic patients and in healthy subjects. *Diabetes* 44:1126-1131, 1995

11. Kieffer TJ, McIntosh CH, Pederson RA: Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. *Endocrinology* 136:3585-3596., 1995

12. Young AA, Gedulin BR, Bhavsar S, Bodkin N, Jodka C, Hansen B, Denaro M: Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (Macaca mulatta). *Diabetes* 48:1026-1034, 1999

13. Greig NH, Holloway HW, De Ore KA, Jani D, Wang Y, Zhou J, Garant MJ, Egan JM: Once daily injection of exendin-4 to diabetic mice achieves long-term beneficial effects on blood glucose concentrations. *Diabetologia* 42:45-50, 1999

14. Kendall DM, Riddle MC, Rosenstock J, Zhuang D, Kim DD, Fineman MS, Baron AD: Effects of exenatide (exendin-4) on glycemic control over 30 weeks in patients with type 2 diabetes treated with metformin and a sulfonylurea. *Diabetes Care* 28:1083-1091, 2005

15. Herman GA, Bergman A, Stevens C, Kotey P, Yi B, Zhao P, Dietrich B, Golor G, Schrodter A, Keymeulen B, Lasseter KC, Kipnes MS, Snyder K, Hilliard D, Tanen M, Cilissen C, De Smet

M, de Lepeleire I, Van Dyck K, Wang AQ, Zeng W, Davies MJ, Tanaka W, Holst JJ, Deacon CF, Gottesdiener KM, Wagner JA: Effect of single oral doses of sitagliptin, a dipeptidyl peptidase-4 inhibitor, on incretin and plasma glucose levels after an oral glucose tolerance test in patients with type 2 diabetes. *J Clin Endocrinol Metab* 91:4612-4619, 2006
16. Raz I, Hanefeld M, Xu L, Caria C, Williams-Herman D, Khatami H: Efficacy and safety of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy in patients with type 2 diabetes mellitus. *Diabetologia* 49:2564-2571, 2006
17. Hansotia T, Baggio LL, Delmeire D, Hinke SA, Yamada Y, Tsukiyama K, Seino Y, Holst JJ, Schuit F, Drucker DJ: Double incretin receptor knockout (DIRKO) mice reveal an essential role for the enteroinsular axis in transducing the glucoregulatory actions of DPP-IV inhibitors. *Diabetes* 53:1326-1335, 2004
18. Baggio LL, Huang Q, Brown TJ, Drucker DJ: Oxyntomodulin and glucagon-like peptide-1 differentially regulate murine food intake and energy expenditure. *Gastroenterology* 127:546-558, 2004
19. Baggio LL, Huang Q, Brown TJ, Drucker DJ: A recombinant human glucagon-like peptide (GLP)-1-albumin protein (albugon) mimics peptidergic activation of GLP-1 receptor-dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis. *Diabetes* 53:2492-2500, 2004
20. Sowden GL, Drucker DJ, Weinshenker D, Swoap SJ: Oxyntomodulin increases intrinsic heart rate in mice independent of the glucagon-like peptide-1 receptor. *Am J Physiol Regul Integr Comp Physiol* 292:R962-970, 2007
21. Baggio LL, Huang Q, Cao X, Drucker DJ: An albumin-exendin-4 conjugate engages central and peripheral circuits regulating murine energy and glucose homeostasis. *Gastroenterology* 134:1137-1147, 2008
22. Malendowicz LK, Nowak KW: Preproglucagon derived peptides and thyrotropin (TSH) secretion in the rat: robust and sustained lowering of blood TSH levels in exendin-4 injected animals. *Int J Mol Med* 10:327-331, 2002
23. Malendowicz LK, Nussdorfer GG, Nowak KW, Ziolkowska A, Tortorella C, Trejter M: Exendin-4, a GLP-1 receptor agonist, stimulates pituitary-adrenocortical axis in the rat: Investigations into the mechanism(s) underlying Ex4 effect. *Int J Mol Med* 12:237-241, 2003
24. Perez-Tilve D, Gonzalez-Matias L, Alvarez-Crespo M, Leiras R, Tovar S, Dieguez C, Mallo F: Exendin-4 potently decreases ghrelin levels in fasting rats. *Diabetes* 56:143-151, 2007
25. Chavez M, Kaiyala K, Madden LJ, Schwartz MW, Woods SC: Intraventricular insulin and the level of maintained body weight in rats. *Behavioral Neuroscience* 109:528-531, 1995
26. Brown LM, Clegg DJ, Benoit SC, Woods SC: Intraventricular insulin and leptin reduce food intake and body weight in C57BL/6J mice. *Physiol Behav* 89:687-691, 2006
27. Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC, Seeley RJ: Hypothalamic mTOR signaling regulates food intake. *Science* 312:927-930, 2006
28. Montrose-Rafizadeh C, Yang H, Rodgers BD, Beday A, Pritchette LA, Eng J: High Potency Antagonists of the Pancreatic Glucagon-like Peptide-1 Receptor. *The Journal of Biological Chemistry* 272:21201-21206, 1997
29. D'Alessio DA, Fujimoto WY, Ensinck JW: Effects of glucagonlike peptide I-(7-36) on release of insulin, glucagon, and somatostatin by rat pancreatic islet cell monolayer cultures. *Diabetes* 38:1534-1538, 1989

30. Seeley RJ, Blake K, Rushing PA, Benoit SC, Eng J, Woods SC, D'Alessio D: The role of CNS GLP-1-(7-36) amide receptors in mediating the visceral illness effects of lithium chloride. *Journal of Neuroscience* 20:1616-1621, 2000
31. Yamamoto H, Lee CE, Marcus JN, Williams TD, Overton JM, Lopez ME, Hollenberg AN, Baggio L, Saper CB, Drucker DJ, Elmquist JK: Glucagon-like peptide-1 receptor stimulation increases blood pressure and heart rate and activates autonomic regulatory neurons. *J Clin Invest* 110:43-52., 2002
32. Rodriquez de Fonseca F, Navarro M, Alvarez E, Roncero I, Chowen JA, Maestre O, Gomez R, Munoz RM, Eng J, Blazquez E: Peripheral versus central effects of glucagon-like peptide-1 receptor agonists on satiety and body weight loss in Zucker obese rats. *Metabolism* 49:709-717, 2000
33. Navarro M, Rodriquez de Fonseca F, Alvarez E, Chowen JA, Zueco JA, Gomez R, Eng J, Blazquez E: Colocalization of glucagon-like peptide-1 (GLP-1) receptors, glucose transporter GLUT-2, and glucokinase mRNAs in rat hypothalamic cells: evidence for a role of GLP-1 receptor agonists as an inhibitory signal for food and water intake. *J Neurochem* 67:1982-1991, 1996
34. Kinzig KP, D'Alessio DA, Seeley RJ: The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. *J Neurosci* 22:10470-10476, 2002
35. Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, Goke B: Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. *J Biol Chem* 268:19650-19655., 1993
36. Fehmann HC, Jiang J, Schweinfurth J, Wheeler MB, Boyd AE, 3rd, Goke B: Stable expression of the rat GLP-I receptor in CHO cells: activation and binding characteristics utilizing GLP-I(7-36)-amide, oxyntomodulin, exendin-4, and exendin(9-39). *Peptides* 15:453-456, 1994
37. Goke R, Larsen PJ, Mikkelsen JD, Sheikh SP: Identification of specific binding sites for glucagon-like peptide-1 on the posterior lobe of the rat pituitary. *Neuroendocrinology* 62:130-134, 1995
38. Goke R, Larsen PJ, Mikkelsen JD, Sheikh SP: Distribution of GLP-1 binding sites in the rat brain: evidence that exendin-4 is a ligand of brain GLP-1 binding sites. *Eur J Neurosci* 7:2294-2300, 1995
39. Baggio LL, Kim JG, Drucker DJ: Chronic exposure to GLP-1R agonists promotes homologous GLP-1 receptor desensitization in vitro but does not attenuate GLP-1R-dependent glucose homeostasis in vivo. *Diabetes* 53 Suppl 3:S205-214, 2004
40. Kolligs F, Fehmann HC, Goke R, Goke B: Reduction of the incretin effect in rats by the glucagon-like peptide 1 receptor antagonist exendin (9-39) amide. *Diabetes* 44:16-19, 1995
41. Barragan JM, Rodriguez RE, Eng J, Blazquez E: Interactions of exendin (9-39) with the effects of GLP-1 (7-36) amide and of exendin-4 on arterial blood pressure and heart rate in rats. *Reg Peptides* 67:63-68, 1996
42. Benito E, Blazquez E, Bosch MA: Glucagon-like peptide-1-(7-36)amide increases pulmonary surfactant secretion through a cyclic adenosine 3',5'-monophosphate-dependent protein kinase mechanism in rat type II pneumocytes. *Endocrinology* 139:2363-2368, 1998
43. Lachey JL, D'Alessio DA, Rinaman L, Elmquist JK, Drucker DJ, Seeley RJ: The role of central glucagon-like peptide-1 in mediating the effects of visceral illness: differential effects in rats and mice. *Endocrinology* 146:458-462, 2005
44. Huo L, Gamber KM, Grill HJ, Bjorbaek C: Divergent leptin signaling in proglucagon neurons of the nucleus of the solitary tract in mice and rats. *Endocrinology* 149:492-497, 2008

45. Montrose-Rafizadeh C, Yang H, Wang Y, Roth J, Montrose MH, Adams LG: Novel signal transduction and peptide specificity of glucagon-like peptide receptor in 3T3-L1 adipocytes. *J Cell Physiol* 172:275-283, 1997
46. Yang H, Egan JM, Wang Y, Moyes CD, Roth J, Montrose MH, Montrose-Rafizadeh C: GLP-1 action in L6 myotubes is via a receptor different from the pancreatic GLP-1 receptor. *Am J Physiol* 275:C675-683, 1998
47. Sancho V, Trigo MV, Gonzalez N, Valverde I, Malaisse WJ, Villanueva-Penacarrillo ML: Effects of glucagon-like peptide-1 and exendins on kinase activity, glucose transport and lipid metabolism in adipocytes from normal and type-2 diabetic rats. *J Mol Endocrinol* 35:27-38, 2005
48. Sancho V, Nuche B, Arnes L, Cancelas J, Gonzalez N, Diaz-Miguel M, Martin-Duce A, Valverde I, Villanueva-Penacarrillo ML: The action of GLP-1 and exendins upon glucose transport in normal human adipocytes, and on kinase activity as compared to morbidly obese patients. *Int J Mol Med* 19:961-966, 2007
49. Gonzalez N, Acitores A, Sancho V, Valverde I, Villanueva-Penacarrillo ML: Effect of GLP-1 on glucose transport and its cell signalling in human myocytes. *Regul Pept* 126:203-211, 2005
50. Arnes L, Gonzalez N, Tornero-Esteban P, Sancho V, Acitores A, Valverde I, Delgado E, Villanueva-Penacarrillo ML: Characteristics of GLP-1 and exendins action upon glucose transport and metabolism in type 2 diabetic rat skeletal muscle. *Int J Mol Med* 22:127-132, 2008
51. Williams DL, Baskin DG, Schwartz MW: Leptin regulation of the anorexic response to glucagon-like peptide-1 receptor stimulation. *Diabetes* 55:3387-3393, 2006
52. Lopez de Maturana R, Donnelly D: The glucagon-like peptide-1 receptor binding site for the N-terminus of GLP-1 requires polarity at Asp198 rather than negative charge. *FEBS Lett* 530:244-248, 2002
53. Lopez de Maturana R, Willshaw A, Kuntzsch A, Rudolph R, Donnelly D: The isolated N-terminal domain of the glucagon-like peptide-1 (GLP-1) receptor binds exendin peptides with much higher affinity than GLP-1. *J Biol Chem* 278:10195-10200, 2003
54. Al-Sabah S, Donnelly D: A model for receptor-peptide binding at the glucagon-like peptide-1 (GLP-1) receptor through the analysis of truncated ligands and receptors. *Br J Pharmacol* 140:339-346, 2003
55. Runge S, Schimmer S, Oschmann J, Schiodt CB, Knudsen SM, Jeppesen CB, Madsen K, Lau J, Thogersen H, Rudolph R: Differential structural properties of GLP-1 and exendin-4 determine their relative affinity for the GLP-1 receptor N-terminal extracellular domain. *Biochemistry* 46:5830-5840, 2007
56. Cabou C, Campistron G, Marsollier N, Leloup C, Cruciani-Guglielmacci C, Penicaud L, Drucker DJ, Magnan C, Burcelin R: Brain glucagon-like peptide-1 regulates arterial blood flow, heart rate, and insulin sensitivity. *Diabetes* 57:2577-2587, 2008
57. Knauf C, Cani PD, Ait-Belgnaoui A, Benani A, Dray C, Cabou C, Colom A, Uldry M, Rastrelli S, Sabatier E, Godet N, Waget A, Penicaud L, Valet P, Burcelin R: Brain glucagon-like peptide 1 signaling controls the onset of high-fat diet-induced insulin resistance and reduces energy expenditure. *Endocrinology* 149:4768-4777, 2008

FIGURE LEGENDS

Figure 1: Comparison of anorectic effects of i3vt GLP-1 and Ex4. A, B: Dose-response curves for i3vt GLP-1 (A) and Ex4 (B). Cumulative 4-hour food intake is shown. C: Time course of anorectic effects of i3vt GLP-1 (3.0 nmol) and Ex4 (0.03 nmol) over 24 hours. D: Preference ratios for 0.1% saccharin vs. total fluid intake during the 4-hour 2-bottle access to saccharin and water. Saccharin was previously paired with i3vt saline, i3vt GLP-1 (3.0 nmol), i3vt Ex4 (0.03 nmol) or ip LiCl (0.15 M administered at 2.0% body weight. Dotted line represents preference ratio for saline-treated rats. Data are represented as mean ± SEM. * $p < 0.05$ vs. saline. # $p < 0.05$ vs. GLP-1.

Figure 2: Effect of GLP-1R antagonists on anorexia induced by i3vt GLP-1 and Ex4. A, B: Rats were pre-treated with i3vt dHEx (10 μg) followed by i3vt GLP-1 (10 μg, A) or Ex4 (0.1 μg, B). C, D: Rats were pre-treated with i3vt Ex9 (100 μg) followed by i3vt GLP-1 (10 μg, C) or Ex4 (0.1 μg, D). Cumulative 4-hour food intake is shown. Data are represented as mean ± SEM. * $p < 0.05$ vs. Sal/Sal. # $p < 0.05$ vs. Sal/GLP-1.

Figure 3: Effect of dHEx on c-Fos IR induced by i3vt GLP-1 and Ex4. Quantification of c-Fos positive nuclei in the PVN (A), NTS (B) and CeA (C) of rats that were treated with i3vt saline or dHEx (10 μg) followed by i3vt saline, GLP-1 (10 μg) or Ex4 (0.1 μg) and sacrificed 2 hours later. Data are represented as mean ± SEM. * $p < 0.05$ vs. Sal/Sal. # $p < 0.05$ vs. Sal/GLP-1.

Figure 4: Effect of dHEx (100 nM) on insulin secretion induced by GLP-1 (1.0 nM) and Ex4 (0.01 and 1.0 nM) in the presence of glucose (200 mg%). Data are represented as mean ± SEM. * $p < 0.05$ vs. Glucose. # $p < 0.05$ vs. Glucose + GLP-1 (1.0 nM) or Glucose + Ex4 (1.0 nM).

Figure 5: Effect of ip dHEx (1.0 mg/kg) on anorexia induced by ip Ex4 (10.0 μg/kg). Cumulative 4-hour food intake is shown. Data are represented as mean ± SEM. * $p < 0.05$ vs. Sal/Sal. # $p < 0.05$ vs. Sal/Ex4.

Figure 6: Effect of i3vt Ex4 in wild-type and GLP-1R-/- mice. A, B: Wild-type mice received i3vt saline or Ex4 (1.0 μg). Food intake (A) and body weight change (B) were measured over 96 hours. C, D: GLP-1R-/- mice received i3vt saline or Ex4 (1.0 μg). Food intake (C) and body weight change (D) were measured over 24 hours. Data are represented as mean ± SEM. * $p < 0.05$ vs. saline.

Figure 1:

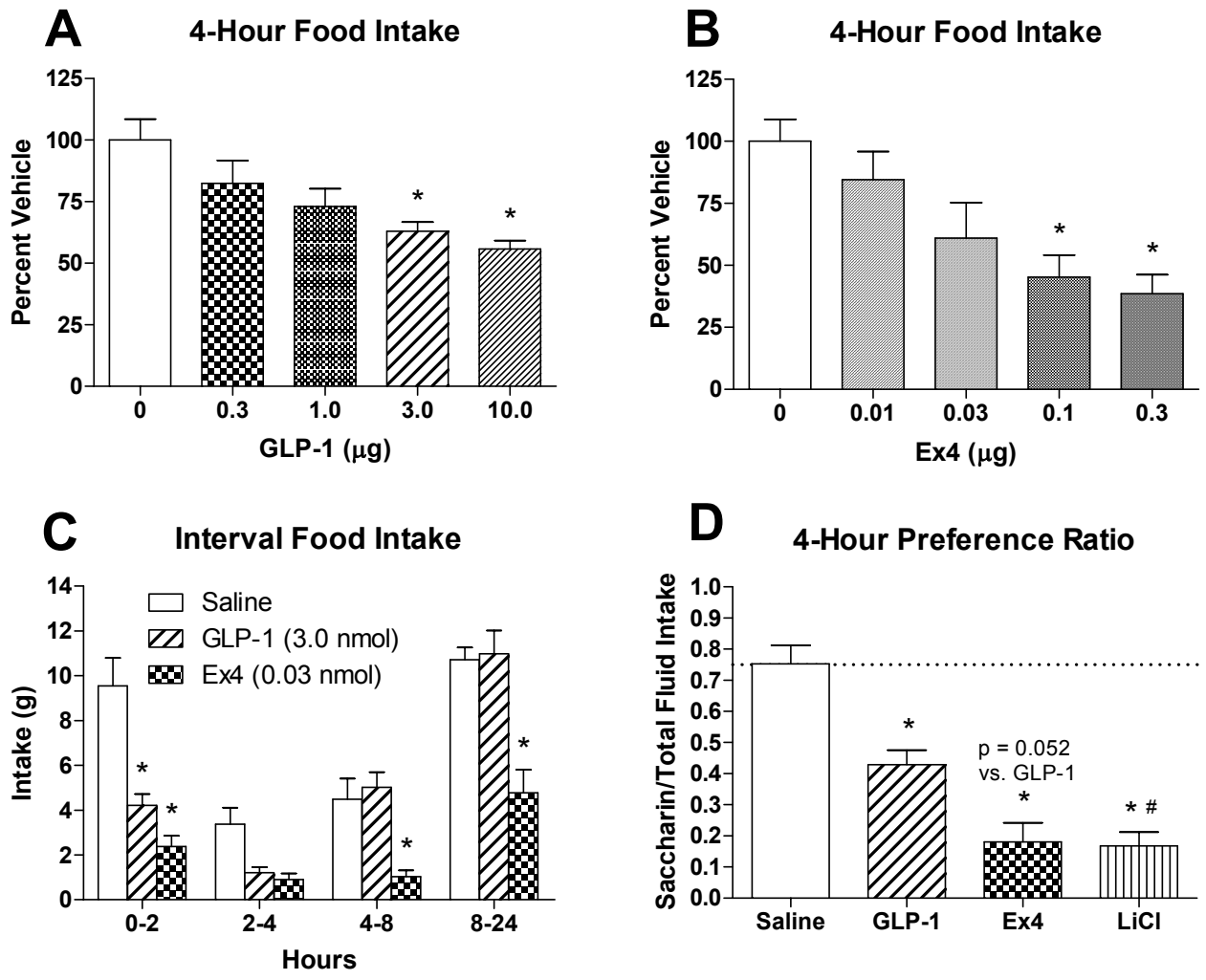

A
4-Hour Food Intake
Percent Vehicle
125
100
75
50
25
0
0
0.3
1.0
3.0
10.0
GLP-1 (μg)
*
*
B
4-Hour Food Intake
Percent Vehicle
125
100
75
50
25
0
0
0.01
0.03
0.1
0.3
Ex4 (μg)
*
*
C
Interval Food Intake
Saline
GLP-1 (3.0 nmol)
Ex4 (0.03 nmol)
Intake (g)
14
12
10
8
6
4
2
0
0-2
2-4
4-8
8-24
Hours
D
4-Hour Preference Ratio
Saccharin/Total Fluid Intake
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
p = 0.052
vs. GLP-1
* #
Saline
GLP-1
Ex4
LiCl

Figure 2:

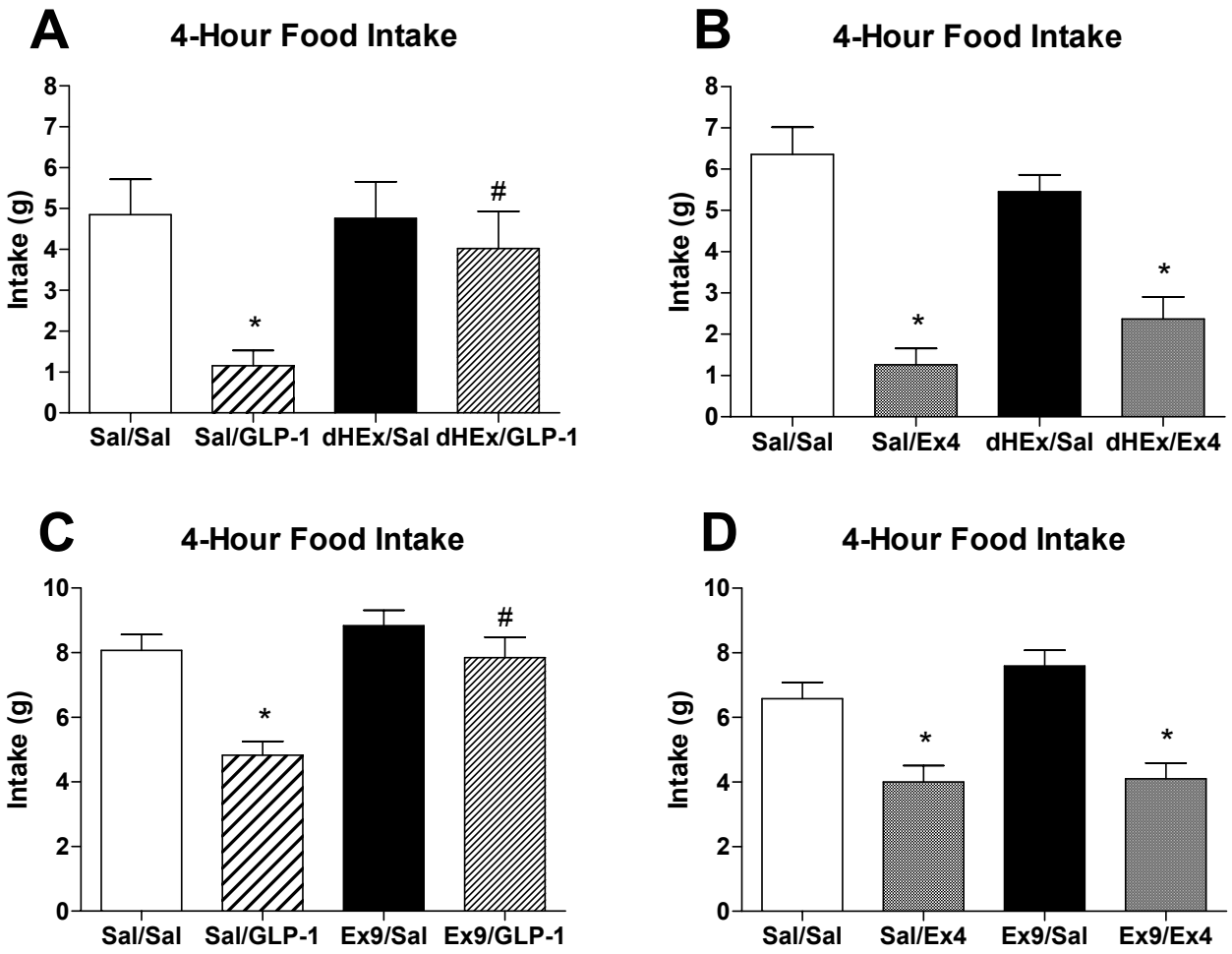

A
4-Hour Food Intake
Intake (g)
Sal/Sal
Sal/GLP-1
dHEx/Sal
dHEx/GLP-1
B
4-Hour Food Intake
Intake (g)
Sal/Sal
Sal/Ex4
dHEx/Sal
dHEx/Ex4
C
4-Hour Food Intake
Intake (g)
Sal/Sal
Sal/GLP-1
Ex9/Sal
Ex9/GLP-1
D
4-Hour Food Intake
Intake (g)
Sal/Sal
Sal/Ex4
Ex9/Sal
Ex9/Ex4

Figure 3:

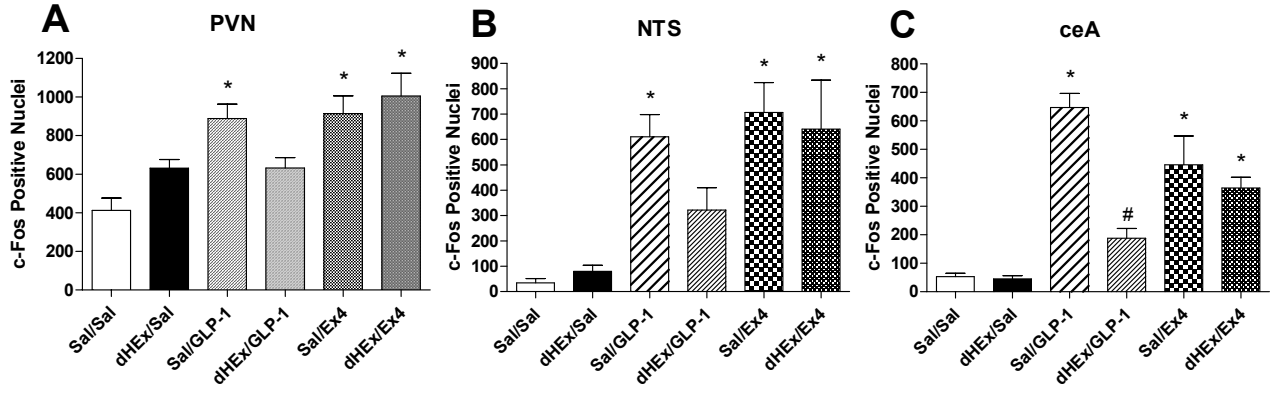


Figure 4:

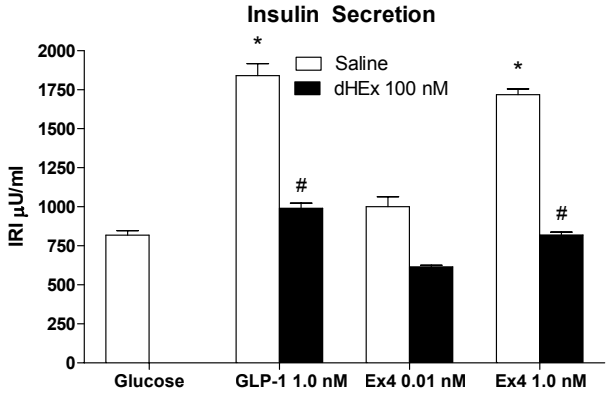


Figure 5:

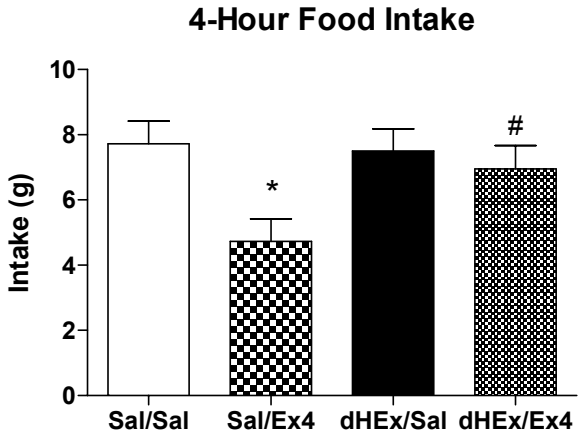
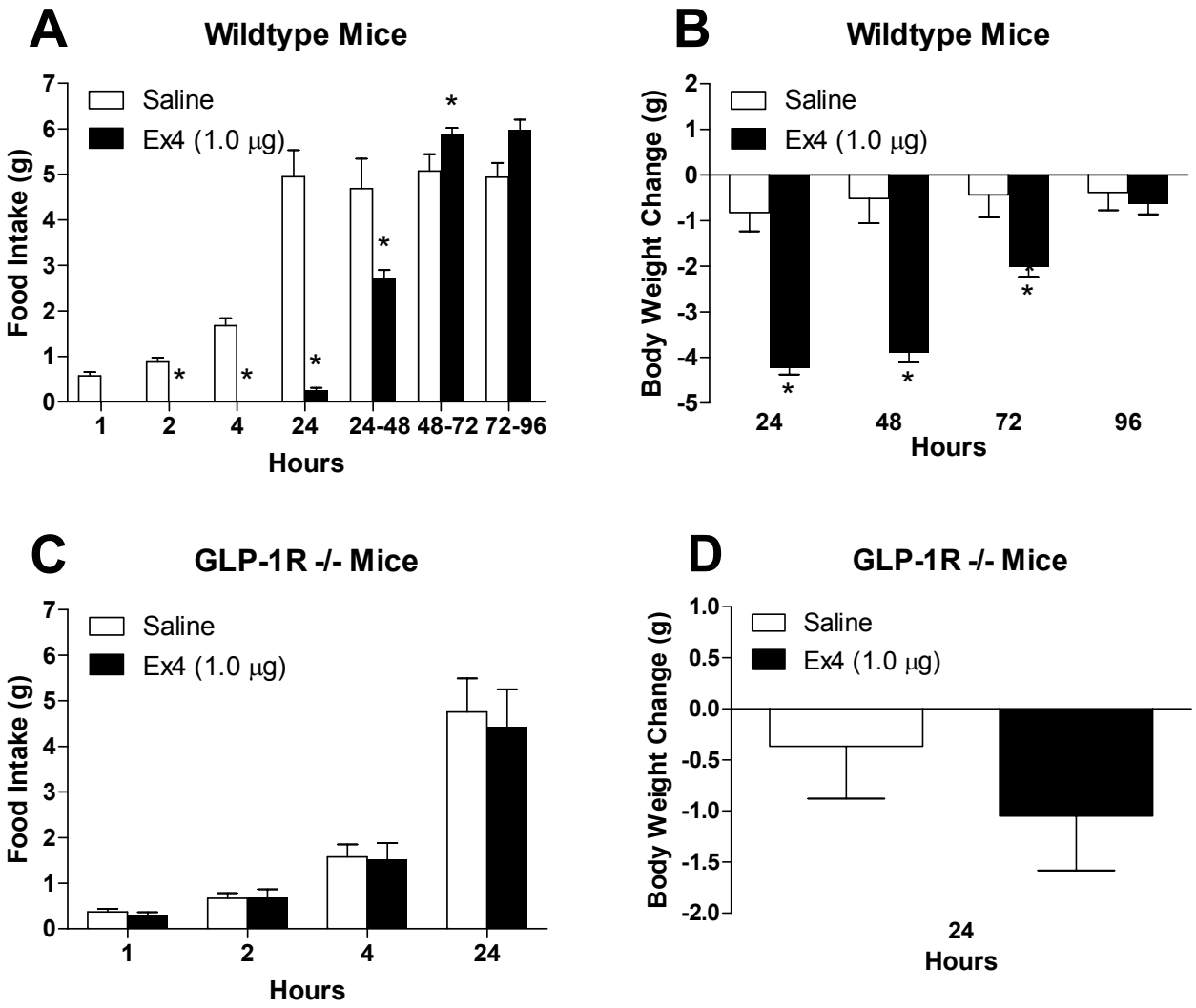


Figure 6:


A
Wildtype Mice
Saline
Ex4 (1.0 μg)
Food Intake (g)
Hours
1
2
4
24
24-48
48-72
72-96
B
Wildtype Mice
Saline
Ex4 (1.0 μg)
Body Weight Change (g)
Hours
24
48
72
96
C
GLP-1R -/- Mice
Saline
Ex4 (1.0 μg)
Food Intake (g)
Hours
D
GLP-1R -/- Mice
Saline
Ex4 (1.0 μg)
Body Weight Change (g)
Hours
24