

Accepted Manuscript

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PII: S0016-5085(08)00056-5
DOI: 10.1053/j.gastro.2008.01.017
Reference: YGAST 54200

To appear in: *Gastroenterology*

Please cite this article as: Baggio, L.L., Huang, Q., Cao, X., Drucker, D.J., An albumin-exendin-4 conjugate engages central and peripheral circuits regulating murine energy and glucose homeostasis, *Gastroenterology* (2008), doi: 10.1053/j.gastro.2008.01.017.

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An albumin-exendin-4 conjugate engages central and peripheral circuits regulating murine energy and glucose homeostasis

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Running title: CJC-1134-PC regulates glucose homeostasis

None of the authors has any current financial relationship with Conjuchem Inc. D. Drucker has previously consulted for Conjuchem Inc during 2007 (one conference call), and consults for companies in the GLP-1/incretin area—a current list of these companies and relationships is disclosed at <http://www.glucagon.com/Drucker%20Lab.htm> These experiments were supported in part by operating grants from the Canadian Diabetes Association, the Juvenile Diabetes Research Foundation and an unrestricted grant from Conjuchem Biotechnologies Inc. D. Drucker receives partial research support through a Canada Research Chair in Regulatory Peptides. Conjuchem Inc had no role in the design, execution, interpretation or writing up of any of the results in this manuscript.

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Background & Aims: Glucagon-like peptide-1 regulates glucose homeostasis through multiple mechanisms including direct actions on the endocrine pancreas, and indirect activation of central nervous system circuits regulating gastric emptying, satiety and body weight. As native GLP-1 is rapidly degraded, there is considerable interest in development of more potent GLP-1 receptor (GLP-1R) agonists with sustained activity however the extent to which much larger GLP-1R agonists will mimic some or all of the actions of smaller peptides remains uncertain.

Methods: We studied the actions of CJC-1134-PC, a recombinant human serum albumin-exendin-4 conjugated protein at the GLP-1 receptor using heterologous cells expressing the GLP-1R in vitro, and both wildtype and *Glp1r*^{-/-} mice in vivo.

Results: CJC-1134-PC activated GLP-1R-dependent signaling in BHK-GLP-1R cells, and acutely lowered blood glucose in WT but not in *Glp1r*^{-/-} mice. Moreover acute administration of CJC-1134-PC rapidly activated c-Fos expression in multiple regions of the central nervous system, acutely inhibited gastric emptying, and produced sustained inhibition of food intake in a GLP-1R-dependent manner. Furthermore, chronic daily treatment of high fat fed wildtype mice with CJC-1134-PC for 4 weeks led to improved glucose tolerance, increased levels of glucose-stimulated insulin, decreased HbA1c and weight loss associated with decreased hepatic triglyceride content.

Conclusions: These findings illustrate that a high molecular weight exendin-4-albumin conjugate retains the ability to mimic a full spectrum of GLP-1R-dependent actions, including activation of CNS circuits regulating gastric emptying, food intake and body weight.

INTRODUCTION

Glucagon-like peptide-1 (GLP-1) is a naturally occurring peptide hormone that is released from intestinal L-cells in response to nutrient ingestion. GLP-1 lowers blood glucose levels in both preclinical studies and in human subjects with type 2 diabetes through multiple distinct actions including stimulation of glucose-dependent insulin secretion, suppression of glucagon secretion, and inhibition of gastric emptying^{1,2}. GLP-1 also promotes satiety, suppresses energy intake and causes weight loss in healthy, obese and diabetic humans. In preclinical studies, GLP-1 increases β -cell mass via induction of β -cell proliferation and neogenesis and inhibition of β -cell apoptosis³⁻⁵. The combined properties of GLP-1 to reduce glycemia and promote weight loss, in association with its potential to restore or sustain β -cell mass and function, have sparked considerable interest in the use of GLP-1 as a therapeutic agent for the treatment of type 2 diabetes (T2DM)⁶.

A major limitation to the therapeutic use of the native GLP-1 molecule is its very short half-life in the circulation, which has been attributed to both rapid cleavage by the ubiquitous proteolytic enzyme dipeptidyl peptidase-4 (DPP-4) and renal clearance. Consequently, GLP-1 must be injected repeatedly or infused continuously to sustain clinical efficacy in vivo. Thus, pharmaceutical strategies based on sustained GLP-1 receptor (GLP-1R) activation have been focused on the development of long-acting DPP-4-resistant GLP-1R agonists such as exenatide and liraglutide. Exenatide is a synthetic version of the lizard salivary gland-derived peptide exendin-4 (Ex-4) that is resistant to DPP-4⁷. Preclinical and clinical studies demonstrate that exenatide is a long-acting GLP-1R agonist^{8,9} and it has been approved for clinical use in the United States and Europe. Liraglutide is a DPP-4-resistant fatty-acylated GLP-1 peptide analog that binds noncovalently to serum albumin following subcutaneous administration, thereby reducing its renal clearance and extending its pharmacokinetic profile¹⁰. Liraglutide exhibits more potent and persistent glucose-lowering effects in diabetic patients compared to native GLP-1 and is currently undergoing evaluation in phase 3 clinical trials in patients with T2DM.

Although exenatide and liraglutide effectively lower blood glucose levels in type 2 diabetic patients, the requirement for twice- or once-daily administration of these agents, respectively, has fostered ongoing efforts to generate GLP-1R agonists with more sustained effectiveness. One such putative agent is CJC-1134-PC, a conjugate consisting of Ex-4 that has been covalently bonded, *ex vivo*, to recombinant human serum albumin via a chemical linker (Supplementary Fig. 1). Preliminary results from clinical trials in diabetic humans indicate that CJC-1134-PC has a half-life of approximately 8 days, making it suitable for once-weekly dosing¹¹. Nevertheless, in short term studies over 4 weeks, CJC-1134-PC produced only modest effects on body weight, hence it remains uncertain whether this high molecular weight GLP-1R agonist is capable of engaging CNS

circuits regulating appetite and body weight. To determine whether the considerably larger Ex-4-albumin hybrid CJC-1134-PC retains the identical spectrum of biological actions exhibited by the much smaller GLP-1R peptide agonist Ex-4, we examined the effects of CJC-1134-PC on GLP-1R-dependent actions *in vitro*, as well as in short- and long-term studies in mice.

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METHODS

Reagents. Cell culture medium was purchased from HyClone (Logan, UT). Serum, G418, and antibiotics were from Invitrogen-Gibco (Burlington, ON). Forskolin, and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma (St. Louis, MO). Human serum albumin (HSA) and CJC-1134-PC were provided by Conjuchem (Montreal, QC). Exendin-4 (Ex-4), glucose-dependent insulintropic peptide (GIP), exendin (9-39), and cholecystokinin (CCK)-8 were purchased from California Peptide Research Inc. (Napa, CA).

Animals. Wild-type C57BL/6 mice (4- or 7-weeks-old) were from Charles River Laboratories (Montreal, QC). Glp1r^{-/-} mice on the CD-1 genetic background have been described¹². Glp1r^{-/-} mice back-crossed onto the C57BL/6 genetic background for six generations¹³ were used in the current studies. All mice were maintained under a 12-hr light/dark cycle (lights on at 7am and off at 7pm) with free access to food and water, except where noted. All experiments were conducted in accordance with protocols and guidelines approved by the University Health Network Animal Care Committee. For acute studies, 8- to 10-week-old wild-type or Glp1r^{-/-} male mice were used. For chronic studies, 4-week-old wild-type male mice were fed either standard rodent chow (SRC) or a high fat diet (HFD, 45% kcal from fat, Research Diets Inc., New Brunswick, NJ) for 4 weeks, then maintained on their respective diets for an additional 4 weeks. Mice on the SRC diet were given twice daily ip injections of phosphate-buffered saline (PBS); HFD mice were randomized to receive: (i) twice daily ip injections of PBS, (ii) twice daily ip injections of Ex-4 (24 nmol/kg), (iii) once daily ip injections of HSA (100 nmol/kg), or (iv) once daily ip injections of CJC-1134-PC. Twice daily injections were at ~ 8 am and 6 pm. For mice receiving once daily injections, drugs were administered in the evening but mice were also given ip injections of PBS in the morning to ensure that all mice were exposed to identical experimental conditions.

Biochemical assays. Blood glucose levels in whole blood were measured using a Glucometer Elite blood glucose meter (Bayer, Toronto, ON). Plasma insulin levels were measured using a Rat/Mouse Insulin ELISA kit (LINCO Research, St. Charles, MO) and pancreatic insulin levels were determined using a Rat insulin RIA kit (LINCO Research, St. Charles, MO). Hb_{A1c} levels were measured using a DCA 2000+ Analyzer (Bayer, Toronto, ON). Plasma glucagon and leptin were measured using a Mouse Endocrine LINCOplex kit (LINCO Research, St. Charles, MO). Serum MCP-1, total PAI-1, and resistin levels were measured using a Mouse Adipokine LINCOplex kit (LINCO Research, St. Charles, MO). Hepatic and/or serum levels of total cholesterol and triglycerides were obtained using specific colorimetric assays (Wako Cholesterol E and Wako L-Type TG-H, Wako Chemicals, Richmond, VA).

In vitro cAMP production. Baby hamster kidney (BHK) fibroblast cells stably transfected with the rat GLP-1R⁵ were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Cells were incubated with 1

μM exendin (9-39) or medium alone for 5 min at 37°C, followed by an additional 10 minute incubation in the presence of increasing concentrations of Ex-4 or CJC-1134-PC. All reactions were carried out in triplicate and terminated by the addition of ice-cold absolute ethanol. cAMP concentration was measured in ethanol extracts using a cAMP RIA kit (Biomedical Technologies, Stoughton, MA).

Glucose tolerance tests. Oral (OGTT) and intraperitoneal (IPGTT) glucose tolerance tests were performed after an overnight fast of 16-18 h after administration of glucose (1.5 mg/g body weight). For acute studies, CJC-1134-PC or HSA was given via ip injection one hour prior to glucose administration, whereas GIP was injected 10 min before glucose administration. For chronic studies, no drugs were administered prior to the glucose tolerance tests.

Feeding studies. Mice were fasted overnight for 16-18h, weighed, and then given ip injections of PBS, or 10 nmol/kg of Ex-4, HSA, or CJC-1134-PC. The mice were then placed into individual cages containing pre-weighed rodent chow with free access to water. Food was removed from the cages at 2, 4, 8 and 24 h following treatment and food intake (g/g body weight) was calculated.

Measurement of gastric emptying rate. The rate of gastric emptying was determined as described in ¹⁴ 4 hours after peptide injections. The gastric emptying rate was calculated using the following equation: gastric emptying rate (%) = [1-(stomach content wet weight/food intake)] x 100.

Evaluation of c-Fos immunoreactivity in the murine CNS. The number of c-Fos immunopositive neurons was quantified in regions of the mouse CNS as described ^{15,16}. Sections were processed for immunocytochemical detection of Fos using an avidin-biotin-immunoperoxidase method (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA) as described ¹⁷. The Fos antibody (Sigma-Aldrich, Oakville, ON) was used at a dilution of 1:50,000. Sections corresponding to CNS regions at the level of the area postrema, nucleus of the solitary tract, and paraventricular nucleus of the hypothalamus were defined according to the Mouse Brain Atlas of Franklin and Paxinos ¹⁸.

Body composition. Total body fat and lean mass were measured using a mouse whole-body magnetic resonance analyzer (Echo Medical Systems, Houston, TX).

β -cell mass determination. Pancreatic sections were immunostained for insulin as described ¹³. Morphometric measurements of insulin-positive area and total pancreatic area were determined using ImageScope software (Aperio Technologies Inc., Vista, CA) and β -cell mass was calculated using the following equation: (insulin positive area/total pancreatic area) x pancreas weight. For each pancreas sample, the β -cell mass was averaged from two different slides that were obtained from the same pancreas sectioned at two different levels.

Hepatic lipids. For histological examination of hepatic lipids, the liver was removed, embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA), frozen rapidly in a dry ice/ethanol bath and stored at -80°C. Frozen tissue was cut into 8 µm sections and stained with oil-red-O using standard protocols. For hepatic lipid determinations, hepatic lipids were extracted as described in ¹⁹.

RNA isolation and real-time PCR. RNA was extracted from pancreas samples using TRI reagent (Sigma, St. Louis, MO) and first-strand cDNA generated using standard protocols. Primers and PCR conditions for the selected genes were those recommended by Applied Biosystems. Relative mRNA levels were quantified using the $2^{-\Delta Ct}$ method ²⁰.

Statistical analysis. All data are presented as means ± SE. Statistical significance was determined by One- or Two-way ANOVA and Bonferroni's post hoc test using Prism version 4.02 software (GraphPad Software, San Diego, CA). A *P* value <0.05 was considered to be statistically significant.

RESULTS

CJC-1134-PC increases cAMP production in vitro. To determine if covalent attachment of a large molecule like HSA impaired the ability of the Ex-4 moiety within CJC-1134-PC to activate the GLP-1R, we compared the potency of CJC-1134-PC versus Ex-4 in vitro. Both Ex-4 and CJC-1134-PC increased cAMP levels in BHK-GLP-1R cells in a dose- and GLP-1R-dependent manner (Supplementary Fig. 2) with similar EC₅₀ values for CJC-1134-PC and Ex-4 (3.47 nM for CJC-1134-PC and 2.62 nM for Ex-4).

CJC-1134-PC mimics the acute actions of Ex-4 on glucose regulation, feeding, and gastric emptying in mice. We next examined if CJC-1134-PC can reproduce the repertoire of actions associated with the smaller Ex-4 peptide. CJC-1134-PC significantly reduced glucose excursion after oral and ip glucose challenge in a dose-dependent manner in wild-type mice (Fig. 1A and C) but not in *Glp1r*^{-/-} mice (Fig. 1E,F) with significant increases in insulin:glucose ratios at doses of 10 and 100 nmol/kg, (Fig. 1B and D). In contrast, GIP potently decreased glucose excursion in *Glp1r*^{-/-} mice (Fig. 1E).

CJC-1134-PC significantly reduced food intake in wild-type mice (Fig. 2) and the effect of CJC-1134-PC and Ex-4 to decrease feeding lasted for at least 24 h (Fig. 2A). Moreover, at 4-8 h and 8-24 h following injection, the reduction in food intake was significantly greater with CJC-1134-PC compared to Ex-4 (Fig. 2). The anorectic action of CJC-1134-PC was not simply a non-specific aversive response as it required a functional GLP-1 receptor (Fig. 2A inset).

The mechanisms through which GLP-1 regulates gastric emptying remain incompletely understood and may require interaction with neural pathways^{21,22}. Both Ex-4 and CJC-1134-PC significantly diminished the gastric emptying rate in wild-type mice (Fig. 2B), but not in *Glp1r*^{-/-} mice (Fig. 2C). In contrast, CCK-8, which inhibits gastric emptying through a distinct mechanism, significantly reduced the gastric emptying rate in *Glp1r*^{-/-} mice (Fig. 2C). To determine if GLP-1R agonists communicate with the CNS to inhibit feeding and gastric emptying, we first compared the effects of peripheral administration of Ex-4 vs. CJC-1134-PC on c-Fos positivity, a marker of neuronal activity (Fig. 3). Ex-4 and CJC-1134-PC produced a similar pattern of c-Fos expression in the mouse CNS that included the area postrema (AP), nucleus of the solitary tract (NTS), and paraventricular nucleus of the hypothalamus (PVH; Fig. 3A, B and C, respectively), regions associated with the control of feeding and gut motility. Moreover, the relative level of c-Fos activation following ip CJC-1134-PC was comparable to that of Ex-4 (Fig. 3A-C). Conversely, ip injection of Ex-4 or CJC-1134-PC failed to increase c-Fos expression in *Glp1r*^{-/-} mice (Fig. 3A-C bar graphs). Immunohistochemical examination of the mouse CNS following ip administration of HSA and did not identify any HSA-immunopositive staining (data not shown). As larger molecules like CJC-1134-PC are unlikely to readily cross the normal blood-brain-barrier,

these data, taken together with the results of previous experiments²³, suggest that GLP-1R agonists do not require direct access to the CNS to modify feeding behaviour and gastric motility.

Chronic administration of CJC-1134-PC improves glucose tolerance and reduces body weight and fat mass in HFD-fed mice. Prolonged administration of GLP-1 or Ex-4 reduces blood glucose levels and promotes weight loss in individuals with type 2 diabetes^{6,24,25}. Accordingly, mice were rendered obese and glucose intolerant and then treated with once- or twice-daily ip injection of CJC-1134-PC or Ex-4, respectively for 4 weeks (Supplementary Fig. 3). Non-fasting blood glucose levels in HFD-fed CJC-1134-PC- and Ex-4-treated mice were lower compared to control mice (Fig. 4A). Mice on the HFD gained more weight compared to mice maintained on the normal chow diet during the first 4 wks (Fig. 4B). However, HFD-fed mice treated with CJC-1134-PC or Ex-4 lost weight and, after 4 weeks of treatment, mice treated with CJC-1134-PC had body weights similar to normal chow-fed PBS-treated mice (Fig. 4B). Serum Hb_{A1c} levels were reduced in HFD-fed mice treated with CJC-1134-PC, but not Ex-4 (Fig. 4C).

Chronic administration of Ex-4 or CJC-1134-PC in HFD-fed mice produced significant reductions in glucose excursions following glucose challenge (Fig. 5A,B and D,E) and CJC-1134-PC but not Ex-4, increased insulin-to-glucose ratios measured following glucose administration (Fig. 5C and F). Chronic treatment with CJC-1134-PC or Ex-4 had no effect on plasma glucagon levels measured in these same samples (data not shown). Both Ex-4 and CJC-1134-PC reduced body weight and fat mass in HFD-fed mice and MRI data demonstrated that Ex-4 and CJC-1134-PC reduced total and percent body fat mass and increased percent lean mass relative to HFD-fed control treated mice (Fig. 6A and B). Furthermore, body weight and epididymal fat pad weights were significantly decreased in CJC-1134-PC- and Ex-4-treated HFD-fed mice, and were comparable to those observed in PBS-treated mice maintained on a normal chow diet (Fig. 6C and data not shown).

CJC-1134-PC or Ex-4 had no effect on serum MCP-1 or PAI-1 levels in HFD-fed mice (Fig. 7A and B), whereas treatment with Ex-4, but not CJC-1134-PC, led to significant reductions in serum resistin (Fig. 7C). Serum leptin levels were significantly lower in both CJC-1134-PC- and Ex-4-treated mice (Fig. 7D). Serum cholesterol levels were reduced in HFD-fed mice following administration of CJC-1134-PC, but not Ex-4 (Fig. 8A). Surprisingly, chronic treatment with both CJC-1134-PC and Ex-4 resulted in significant elevations in serum triglyceride levels (Fig. 8B). Although hepatic cholesterol levels were unchanged (data not shown), total hepatic triglyceride content was reduced following treatment with CJC-1134-PC or Ex-4 in HFD-fed mice (Fig. 8C). Oil-Red O staining (Supplementary Fig. 4) further illustrated the reduced hepatic lipid content in CJC-1134-PC- and Ex-4-treated mice. The decrease in hepatic triglyceride, in association with increased

plasma triglyceride levels, raised the possibility that mice treated with CJC-1134-PC or Ex-4 fail to accumulate triglyceride in the liver, perhaps leading to increased circulating levels of triglycerides. Hence, we examined the expression of hepatic genes whose products are important for lipid absorption, transport, metabolism or synthesis. Real-time quantitative PCR demonstrated that treatment with CJC-1134-PC or Ex-4 was associated with significantly reduced levels of mRNA transcripts for fatty acid binding proteins 1 and 2 and CD36, respectively (Fig. 8D-F). Conversely, CJC-1134-PC or Ex-4 administration did not alter the mRNA levels of apolipoprotein B, hepatic lipase, PPAR α or Srebf1 (data not shown).

Chronic administration of CJC-1134-PC up-regulates expression of β -cell specific genes and increases pancreas weight but has no effect on β -cell mass in HFD-fed mice.

Ex-4, but not CJC-1134-PC, increased insulin mRNA levels compared to saline-treated mice (Supplementary Fig. 5A). In contrast, pancreas insulin content was significantly reduced in CJC-1134-PC-treated mice relative to levels in mice treated with HSA but was not significantly different in Ex-4- vs. control-treated mice (Supplementary Fig. 5B). Although both CJC-1134-PC and Ex-4 increased pancreas weights in HFD-fed mice (Supplementary Fig. 5C), no significant increases in β -cell mass were observed following chronic treatment with either agent (Supplementary Fig. 5D). GLP-1R agonists also increase the expression of genes that contribute to β -cell glucose sensing, proliferation, neogenesis or cytoprotection^{2, 26, 27}. mRNA levels of *Gck*, *Glp1r*, and *Pdx1* were increased in pancreatic RNA from both CJC-1134-PC- and/or Ex-4-treated mice. (Supplementary Fig. 5E-G). Surprisingly however, Ex-4, but not CJC-1134-PC, markedly increased levels of mRNA transcripts for *Irs2* (Supplementary Figure 5H).

DISCUSSION

Although there is much interest in developing GLP-1-based strategies for the treatment of T2DM^{1,6}, its short half-life in the circulation has posed practical limitations on the clinical use of the native peptide. Exenatide is the first DPP-4 resistant GLP-1R agonist approved for the treatment of T2DM. However, the need for twice-daily injections of exenatide has spawned efforts to generate GLP-1R agonists with more prolonged pharmacokinetic and pharmacodynamic properties. To this end, albumin binding has been utilized as a strategy to prolong GLP-1R agonist action *in vivo*, as exemplified by the interaction of acylated liraglutide with albumin, resulting in a circulating half-life of ~11-15 h in humans²⁸. Nevertheless, as liraglutide interacts with albumin in a non-covalent manner, and is also cleared by the kidney it requires once daily administration to achieve continuous GLP-1R activation.

CJC-1134-PC is distinct from liraglutide in that it is a preformed conjugate consisting of Ex-4 that has been covalently bound *ex vivo* to recombinant human serum albumin via a chemical linker. Our experimental data, encompassing receptor activation studies *in vitro*, and acute and chronic experiments *in vivo*, demonstrate that the larger albumin-Ex-4 conjugate reproduces the majority of biological actions associated with the much smaller GLP-1R peptide agonist Ex-4.

Surprisingly, despite its larger size, CJC-1134-PC was equipotent to Ex-4 in its ability to stimulate cAMP production in contrast to Albugon (Albiglutide), a recombinant GLP-1-albumin fusion protein which exhibited reduced affinity and potency for the GLP-1R²³. Albugon contains the peptide sequences of GLP-1 within the open reading frame of recombinant human serum albumin whereas CJC-1134-PC contains an Ex-4 molecule linked covalently to a single cysteine residue on human serum albumin. We suspect that the Ex-4 moiety within CJC-1134-PC is less constrained and likely more mobile than the GLP-1 epitope within Albugon, perhaps accounting for the marked difference in potency at the GLP-1R. Hence, one might predict that therapy with CJC-1134-PC may require lower circulating levels relative to treatment with Albugon, a hypothesis that may be tested in future studies. Consistent with the results of receptor studies, CJC-1134-PC and Ex-4 were administered at equimolar doses in the feeding study and CJC-1134-PC was as effective as Ex-4 at reducing food intake. Hence the larger molecular weight CJC-1134-PC is as potent an inhibitor of GLP-1R-dependent feeding as the small Ex-4.

Small GLP-1R agonists like GLP-1 and Ex-4 inhibit feeding and gastric emptying either by direct activation of CNS centers or indirectly, through activation of vagal afferent pathways^{21,29,30}. As large proteins like albumin cannot readily cross an intact blood-brain barrier³¹, the ability of CJC-1134-PC to inhibit food intake and gastric emptying suggests that GLP-1R agonists do not require direct access to the CNS to mediate

these effects, and are in agreement with data demonstrating that GLP-1 and/or Ex-4 modify feeding and gut motility via the vagus^{21, 32, 33}. These results are corroborated by the demonstration that peripherally administered CJC-1134-PC activates c-fos expression in the murine CNS, and produces weight loss in mice. Hence, high molecular weight GLP-1R agonists such as CJC-1134-PC remain capable of activating circuits regulating gastric emptying and satiety in a GLP-1R-dependent manner.

In humans, GLP-1 or Ex-4 treatment has been associated with significant reductions in post-prandial plasma triglyceride levels whereas chronic treatment with either CJC-1134-PC or Ex-4 led to significantly increased non-fasting plasma triglyceride levels but reduced hepatic lipid content. These results highlight key differences between the control of murine vs. human hepatic lipid homeostasis. Real-time PCR analysis revealed that CJC-1134-PC- and Ex-4-treatment reduced hepatic levels of genes that encode proteins involved in hepatic lipid uptake and intracellular transport (CD36, Fabp1, Fabp2), suggesting that lipids are being diverted from the liver.

Although demonstrated that GLP-1R agonists increase β -cell mass in diabetic rodents, we detected no increase in β -cell mass in HFD-fed mice that were treated chronically with CJC-1134-PC or Ex-4. Since HFD-feeding can lead to either no changes or increases in β -cell mass to compensate for HFD-induced insulin resistance^{34, 35}, it is possible that the replicative capacity of the β -cell is maximized in our studies and cannot be further increased with CJC-1134-PC or Ex-4. Remarkably, although both Ex-4 and CJC-1134 increased levels of mRNA transcripts for the *Glp1r*, *Gck*, and *Pdx1*, only Ex-4 robustly increased levels of *Irs2*, a GLP-1R-activated signaling protein critically important for β -cell survival³⁶. The explanation for this difference in gene expression profiles remains unclear, but suggests that structurally different GLP-1R agonists may exhibit unique patterns of GLP-1R activation in different tissues.

Taken together, our findings provide additional evidence supporting the notion that high molecular weight GLP-1R agonists can activate a broad spectrum of GLP-1R-dependent actions. Notably, CJC-1134-PC potently activated c-Fos in multiple regions of the CNS, reduced gastric emptying and food intake in acute studies, and promoted weight loss in chronic experiments. These findings extend our understanding of the central and peripheral actions of GLP-1R agonists with implications for the predicted use and efficacy of these new high molecular weight proteins for the treatment of T2DM.

1. Deacon CF. Therapeutic strategies based on glucagon-like peptide 1. *Diabetes* 2004;53:2181-2189.
2. Drucker DJ. The biology of incretin hormones. *Cell Metab* 2006;3:153-165.
3. Xu G, Stoffers DA, Habener JF, et al. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 1999;48:2270-2276.
4. Stoffers DA, Kieffer TJ, Hussain MA, et al. Insulinotropic glucagon-like peptide-1 agonists stimulate expression of homeodomain protein IDX-1 and increase β -cell mass in mouse pancreas. *Diabetes* 2000;49:741-748.
5. Li Y, Hansotia T, Yusta B, et al. Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. *J Biol Chem* 2003;278:471-478.
6. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* 2006;368:1696-1705.
7. Eng J, Kleinman WA, Singh L, et al. Isolation and characterization of exendin 4, an exendin 3 analogue from *Heloderma suspectum* venom. *J Biol Chem* 1992;267:7402-7405.
8. Young AA, Gedulin BR, Bhavsar S, et al. 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* 1999;48:1026-1034.
9. Kolterman OG, Buse JB, Fineman MS, et al. Synthetic exendin-4 (exenatide) significantly reduces postprandial and fasting plasma glucose in subjects with type 2 diabetes. *J Clin Endocrinol Metab* 2003;88:3082-3089.
10. Agero H, Jensen LB, Elbrond B, et al. The pharmacokinetics, pharmacodynamics, safety and tolerability of NN2211, a new long-acting GLP-1 derivative, in healthy men. *Diabetologia* 2002;45:195-202.
11. Wang M, Kipnes MS, Matheson S, et al. Safety and Pharmacodynamics of CJC-1134-PC, a Novel GLP-1 Receptor Agonist, in Patients with Type 2 Diabetes Mellitus: A Randomized, Placebo-Controlled, Double-Blind, Dose-Escalation Study, In American Diabetes Association, Chicago, 2007.
12. Scrocchi LA, Brown TJ, MacLusky N, et al. Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide receptor gene. *Nature Med* 1996;2:1254-1258.
13. Hansotia T, Maida A, Flock G, et al. Extrapancreatic incretin receptors modulate glucose homeostasis, body weight, and energy expenditure. *J Clin Invest* 2007;117:143-152.

14. Asakawa A, Inui A, Yuzuriha H, et al. Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. *Gastroenterology* 2003;124:1325-1336.
15. Elias CF, Kelly JF, Lee CE, et al. Chemical characterization of leptin-activated neurons in the rat brain. *J Comp Neurol* 2000;423:261-281.
16. Yamamoto H, Kishi T, Lee CE, et al. Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites. *J Neurosci* 2003;23:2939-2946.
17. Yamamoto H, Lee CE, Marcus JN, et al. Glucagon-like peptide-1 receptor stimulation increases blood pressure and heart rate and activates autonomic regulatory neurons. *J Clin Invest* 2002;110:43-52.
18. Franklin K, Paxinos G. *The mouse brain in stereotaxic coordinates*. Academic Press, 1997.
19. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957;226:497-509.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-408.
21. Imeryuz N, Yegen BC, Bozkurt A, et al. Glucagon-like peptide-1 inhibits gastric emptying via vagal afferent-mediated central mechanisms. *Am J Physiol* 1997;273:G920-G927.
22. Nagell CF, Wettergren A, Orskov C, et al. Inhibitory effect of GLP-1 on gastric motility persists after vagal deafferentation in pigs. *Scand J Gastroenterol* 2006;41:667-672.
23. Baggio LL, Huang Q, Brown TJ, et al. 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* 2004;53:2492-2500.
24. Zander M, Madsbad S, Madsen JL, et al. Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. *Lancet* 2002;359:824-830.
25. DeFronzo RA, Ratner RE, Han J, et al. Effects of Exenatide (Exendin-4) on Glycemic Control and Weight Over 30 Weeks in Metformin-Treated Patients With Type 2 Diabetes. *Diabetes Care* 2005;28:1092-1100.
26. Wang YH, Egan JM, Raygada M, et al. Glucagon-like peptide-1 affects gene transcription and messenger ribonucleic acid stability of components of the insulin secretory system in RIN 1046-38 cells. *Endocrinology* 1995;136:4910-4917.

27. Drucker DJ. Glucagon-like peptides: regulators of cell proliferation, differentiation, and apoptosis. *Mol Endocrinol* 2003;17:161-171.
28. Elbrond B, Jakobsen G, Larsen S, et al. Pharmacokinetics, pharmacodynamics, safety, and tolerability of a single-dose of NN2211, a long-acting glucagon-like peptide 1 derivative, in healthy male subjects. *Diabetes Care* 2002;25:1398-1404.
29. 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* 2002;22:10470-10476.
30. Hellstrom PM, Naslund E. Interactions between gastric emptying and satiety, with special reference to glucagon-like peptide-1. *Physiol Behav* 2001;74:735-741.
31. Chuang VT, Kragh-Hansen U, Otagiri M. Pharmaceutical strategies utilizing recombinant human serum albumin. *Pharm Res* 2002;19:569-577.
32. Abbott CR, Monteiro M, Small CJ, et al. The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. *Brain Res* 2005;1044:127-131.
33. Talsania T, Anini Y, Siu S, et al. Peripheral exendin-4 and peptide YY(3-36) synergistically reduce food intake through different mechanisms in mice. *Endocrinology* 2005;146:3748-3756.
34. Andrikopoulos S, Massa CM, Aston-Mourney K, et al. Differential effect of inbred mouse strain (C57BL/6, DBA/2, 129T2) on insulin secretory function in response to a high fat diet. *J Endocrinol* 2005;187:45-53.
35. Hull RL, Andrikopoulos S, Verchere CB, et al. Increased dietary fat promotes islet amyloid formation and beta-cell secretory dysfunction in a transgenic mouse model of islet amyloid. *Diabetes* 2003;52:372-379.
36. Jhala US, Canettieri G, Sreaton RA, et al. cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. *Genes Dev* 2003;17:1575-1580.

Figure 1. CJC-1134-PC reduces glucose excursions in wild-type but not *Glp1r*^{-/-} mice. (A) Oral (OGTT) and (C) intraperitoneal (IPGTT) glucose tolerance in wild-type mice following ip administration of different doses (nmol/kg) of CJC-1134-PC or human serum albumin (HSA) 60 min prior to glucose loading. ****P*<0.001 for AUC data (not shown) for CJC-1134-PC- vs. HSA-treated mice at each dose. (B and D) Plasma insulin-to-glucose ratios at the 10-20 min time point after glucose administration in wild-type mice treated with the indicated doses of CJC-1134-PC or HSA. (E) Oral glucose tolerance and (F) AUC glucose in *Glp1r*^{-/-} mice following ip administration of 100 nmol/kg CJC-1134-PC and HSA or 3 μ g of glucose-dependent insulinotropic peptide (GIP) at 60 min (CJC-1134-PC and HSA) or 10 min (GIP) prior to oral glucose challenge. Values are expressed as means \pm SE; n = 4-11 mice/group. ***P*<0.01, ****P*<0.001 for CJC-1134-PC- vs. HSA-treated mice.

Figure 2. CJC-1134-PC inhibits feeding and gastric emptying rate in wild-type but not *Glp1r*^{-/-} mice. (A) Following an overnight fast, wild-type or *Glp1R*^{-/-} mice were give ip injections of PBS or 10 nmol/kg of exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC and food intake was determined at 2, 4, 8 and 24 h post injection. Gastric emptying rate in (B) wild-type and (C) *Glp1r*^{-/-} mice at 4 h after ip administration of PBS, Ex-4 (24 nmol/kg), HSA (100 nmol/kg), CJC-1134-PC (100 nmol/kg), or cholecystokinin-8 (CCK-8; 3 μ g). Values are expressed as means \pm SE; n = 3-6 mice/group. ##*P*<0.01, ####*P*<0.001 for Ex-4- vs. PBS-treated mice. ****P*<0.001 for CJC-1134-PC- vs. HSA-treated mice. +++*P*<0.001 for CJC-1134-PC- vs. Ex-4-treated mice.

Figure 3. Peripheral administration of CJC-1134-PC increases c-FOS expression

Representative photomicrographs of c-FOS stained mouse coronal brain sections of (A) area postrema (AP), (B) nucleus of the solitary tract (NTS), and (C) paraventricular nucleus of the hypothalamus (PVH) in wild-type mice at 60 min following ip injection of PBS, exendin-4 (Ex-4; 24 nmol/kg), human serum albumin (HSA; 100 nmol/kg), or CJC-1134-PC (100 nmol/kg). Original magnification, x 200. CC, central canal; 3V, third ventricle. The number of c-fos⁺ cells in wild-type and *Glp1r*^{-/-} mice is indicated below the corresponding CNS section. Data are presented as means \pm SE; n = 3-5 mice/group. ##*P*<0.01, ####*P*<0.001 for Ex-4- vs. PBS-treated mice. ***P*<0.01, ****P*<0.001 for CJC-1134-PC- vs. HSA-treated mice.

Figure 4. CJC-1134-PC reduces body weight, non-fasting blood glucose and Hb_{A1c} in HFD-fed wild-type mice. WT mice were placed on a high-fat diet (HFD) for 4 wks and then treated for 4 weeks with daily ip injections of 100 nmol/kg of human serum albumin (HSA) or CJC-1134-PC or twice-a-day injections of PBS or exendin-4 (Ex-4; 24 nmol/kg). Separate groups of mice maintained on regular chow throughout the entire 8 wk study (RC PBS) were given ip injections of PBS twice-a-day. The change in (A) non-fasting blood glucose and (B) body weight from baseline was determined weekly. (C) %Hb_{A1c} in whole blood after 4 wks of treatment with PBS, Ex-4, HSA, or CJC-1134-PC in HFD-fed wild-type mice or in regular chow-fed mice treated with PBS (RC PBS). Data represent means \pm SE; n = 7-8 mice/group. ** P <0.01 for CJC-1134-PC- vs. HSA-treated mice.

Figure 5. CJC-1134-PC improves glucose tolerance in HFD-fed wild-type mice. (A and D) glucose excursion, (B and E) AUC glucose, and (C and F) plasma insulin-to-glucose ratios during an intraperitoneal (IPGTT) or oral (OGTT) glucose tolerance test in high fat diet (HFD)-fed wild-type mice treated chronically with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC or in mice maintained on normal chow and treated chronically with PBS (RC PBS). Plasma insulin-to-glucose ratios were determined at the 10-20 min time point following glucose challenge. Values are expressed as means \pm SE; n = 7-8 mice/group. ### P <0.01, #### P <0.001 for Ex-4- vs. PBS-treated mice. * P <0.05, *** P <0.001 for CJC-1134-PC- vs. HSA-treated mice.

Figure 6. CJC-1134-PC decreases body weight and fat mass in HFD-fed wild-type mice. MRI determinations of (A) fat mass and (B) lean mass, expressed as total (g) or normalized to body weight (%), in high fat diet (HFD)-fed wild-type mice after 2 wks of treatment with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC or in mice maintained on normal chow and treated with PBS (RC PBS) for 2 wks. (C) Body weight in HFD-fed mice after 4 wks of treatment with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC or in mice maintained on normal chow and treated with PBS (RC PBS). Data are expressed as means \pm SE; n = 7-8 mice/group. ### P <0.01, #### P <0.001 for Ex-4- vs. PBS-treated mice. ** P <0.01, *** P <0.001 for CJC-1134-PC- vs. HSA-treated mice.

Figure 7. CJC-1134-PC reduces resistin and leptin levels in HFD-fed wild-type mice. Serum levels of (A) MCP-1, (B) PAI-1, (C) resistin, and (D) leptin were assayed after 4 wks of treatment with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC in high fat diet (HFD)-fed wild-type mice or in regular

chow-fed mice treated with PBS (RC PBS). Data are expressed as means \pm SE; $n = 4-8$ mice/group. $##P < 0.01$, $###P < 0.001$ for Ex-4- vs. PBS-treated mice. $***P < 0.001$ for CJC-1134-PC- vs. HSA-treated mice.

Figure 8. Serum triglyceride levels are increased but hepatic triglyceride and mRNA levels of fatty acid transport proteins are reduced in mice treated with CJC-1134-PC. Serum cholesterol (A) and, triglyceride (B) levels and hepatic triglyceride content (C) in high fat diet (HFD)-fed wild-type mice treated chronically with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC or in mice maintained on normal chow and treated chronically with PBS (RC PBS). Data are means \pm SE; $n = 6-7$ mice/group. Relative hepatic mRNA levels of (D) Fabp1, (E) Fabp2, and (F) CD36 determined by real-time quantitative PCR in HFD-fed wild-type after chronic treatment with PBS, Ex-4, HSA, or CJC-1134-PC or in RC-fed mice treated with PBS. Data are means \pm SE and are normalized to 18S rRNA; $n = 3-4$ mice/group. For A-F, $\#P < 0.05$, $##P < 0.01$, $###P < 0.001$ for Ex-4- vs. PBS-treated mice and $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ for CJC-1134-PC- vs. HSA-treated mice.

Supplementary figure 1. Structure of CJC-1134-PC. CJC-1134-PC is comprised of a synthetically modified exendin-4 derivative and recombinant human serum albumin. The exendin-4 derivative includes amino acids 1 to 39 of native exendin-4 and a lysine residue at position 40. This lysine is covalently bonded to the cysteine 34 residue of recombinant human serum albumin via an amino-ethoxy-ethoxy-acetyl linker and a reactive maleimidopropionic acid group.

Supplementary figure 2. CJC-1134-PC exhibits similar potency to Ex-4 at the rat GLP-1 receptor in vitro. BHK cells stably expressing the rat GLP-1R were pretreated for 5 min with 1 μ M exendin (9-39) [9-39] or medium alone prior to a 10 min treatment with increasing concentrations of CJC-1134-PC or exendin-4 (Ex-4). cAMP levels in desiccated aliquots of cells plus media extracts were measured by radioimmunoassay and used to calculate the total cAMP content per well. Values are data from an experiment performed in triplicate.

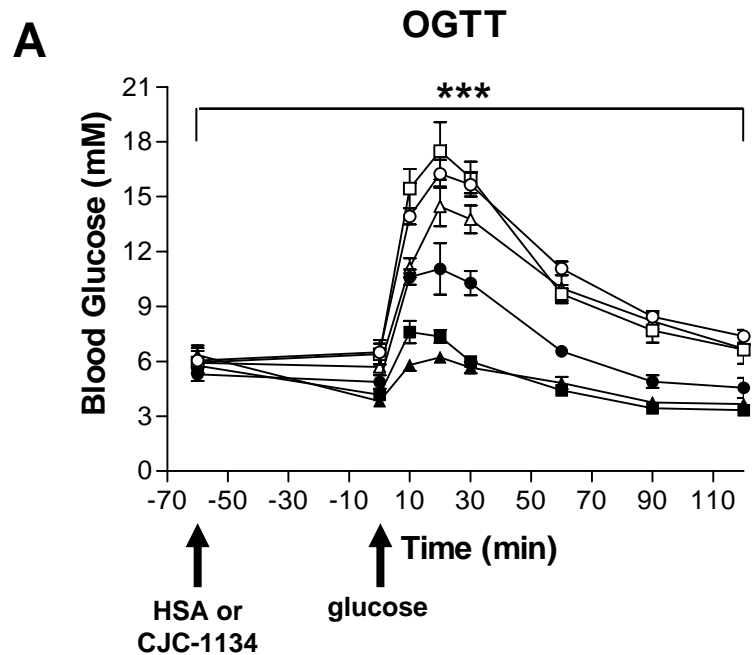
Supplementary figure 3. Schematic depiction of chronic studies. Wild-type mice were fed a high fat diet (HFD) for 4 wks and then treated (Tx) with once- or twice-daily ip injection of PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC at the indicated doses for 4 wks. A separate group of mice was maintained on a normal chow diet for 4 wks and treated with twice daily ip injections of PBS for 4 wks. Non-fasting blood glucose levels and body weights were measured weekly. Body composition was evaluated by MRI after 2 wks of treatment. Intraperitoneal (IPGTT) and oral (OGTT) glucose tolerance were assessed after 3 and 4 wks of treatment, respectively. After 4 weeks of treatment, mice were sacrificed and various end-points determined.

Supplementary figure 4. Oil-red O staining of hepatic tissue. Representative photomicrographs of oil-red O-stained liver tissue from HFD-fed mice after 4 wks of treatment with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC. Original magnification, x 200.

Supplementary figure 5. Chronic treatment with CJC-1134-PC increases β -cell-specific gene transcription and increases pancreas weight but does not affect β -cell mass in HFD-fed mice. (A) relative levels of pancreas insulin mRNA, (B) pancreas insulin content, (C) pancreas weight normalized to body weight, and (D) beta cell mass in high fat diet (HFD)-fed wild-type mice treated chronically with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC or in mice maintained on normal chow and treated chronically with PBS (RC PBS). Values represent means \pm SE; n = 4-8 mice/group. Relative pancreatic mRNA levels of (E) Glp1R, (F) glucokinase, (G) PDX-1, and (H) IRS-2 determined by real-time quantitative PCR in HFD-fed wild-type after chronic treatment with PBS, Ex-4, HSA, or CJC-1134-PC or in RC-fed mice treated with PBS. Data are means \pm SE; n = 3-4 mice/group. For A and E-F, data are normalized to islet

amyloid polypeptide mRNA content. For A-H, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ for Ex-4- or GIP- vs. PBS-treated mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for CJC-1134-PC- vs. HSA-treated mice. +++ $P < 0.001$ for CJC-1134-PC- vs. Ex-4-treated mice.

ACCEPTED MANUSCRIPT



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 CJC-1134 (1)
 HSA (10)
 CJC-1134 (10)
 HSA (100)
 CJC-1134 (100)

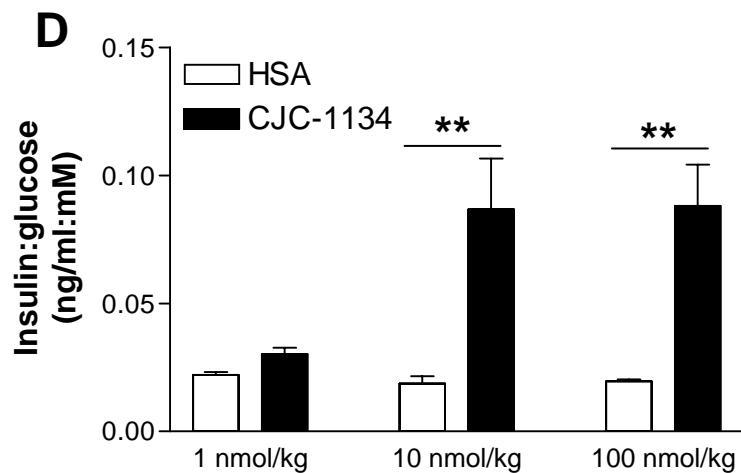
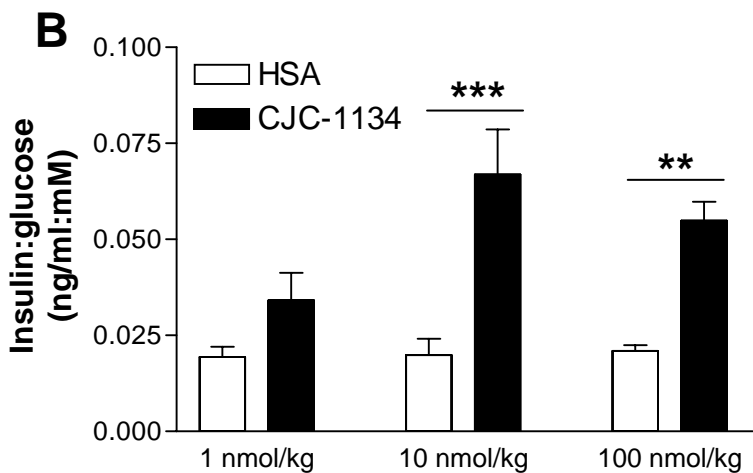
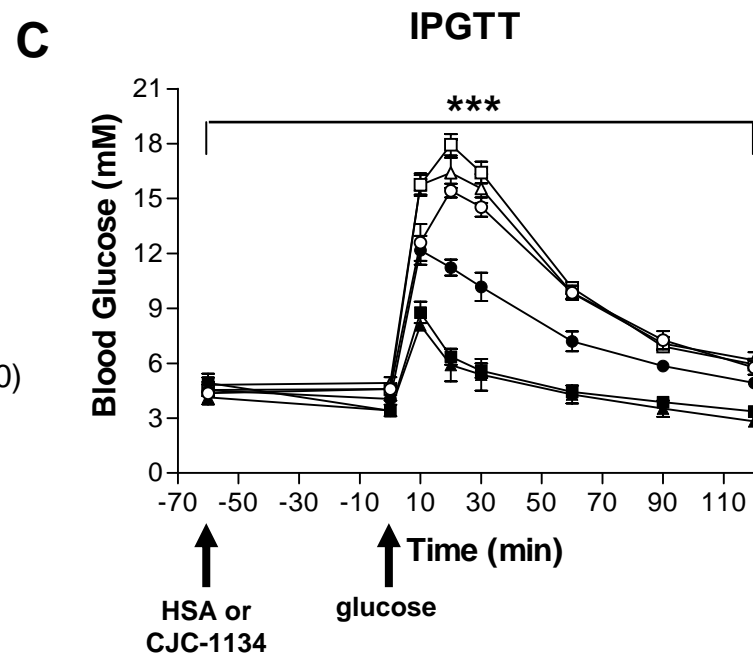
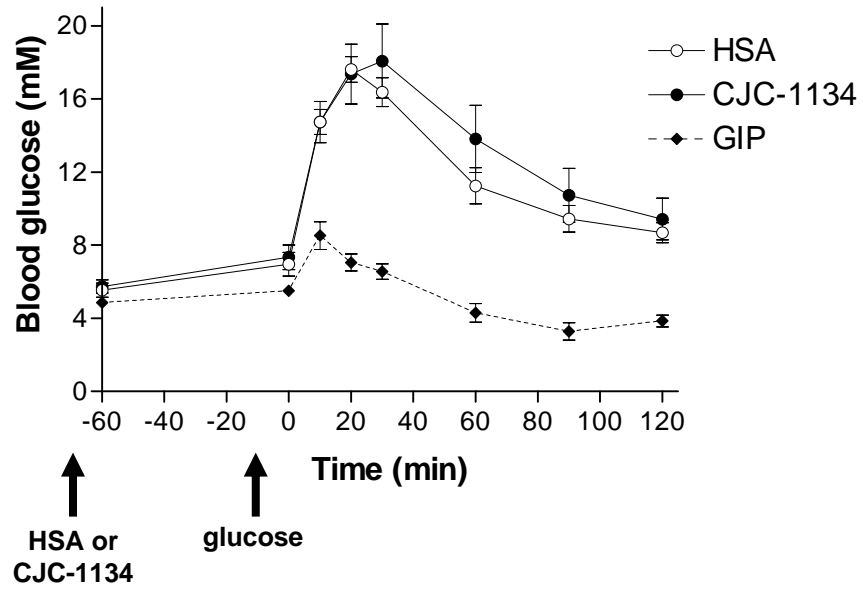
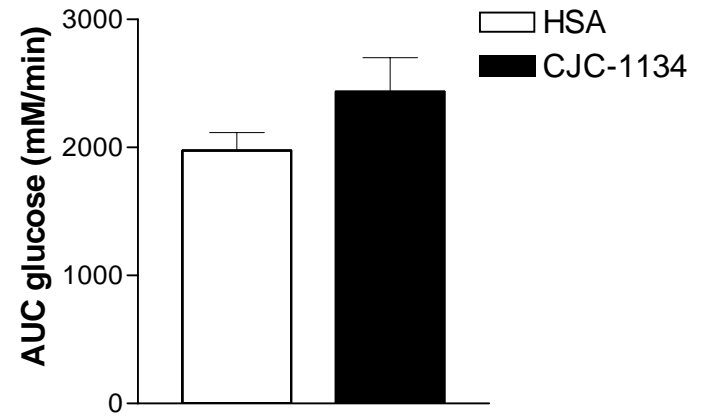


Figure 1

E**F****Figure 1**

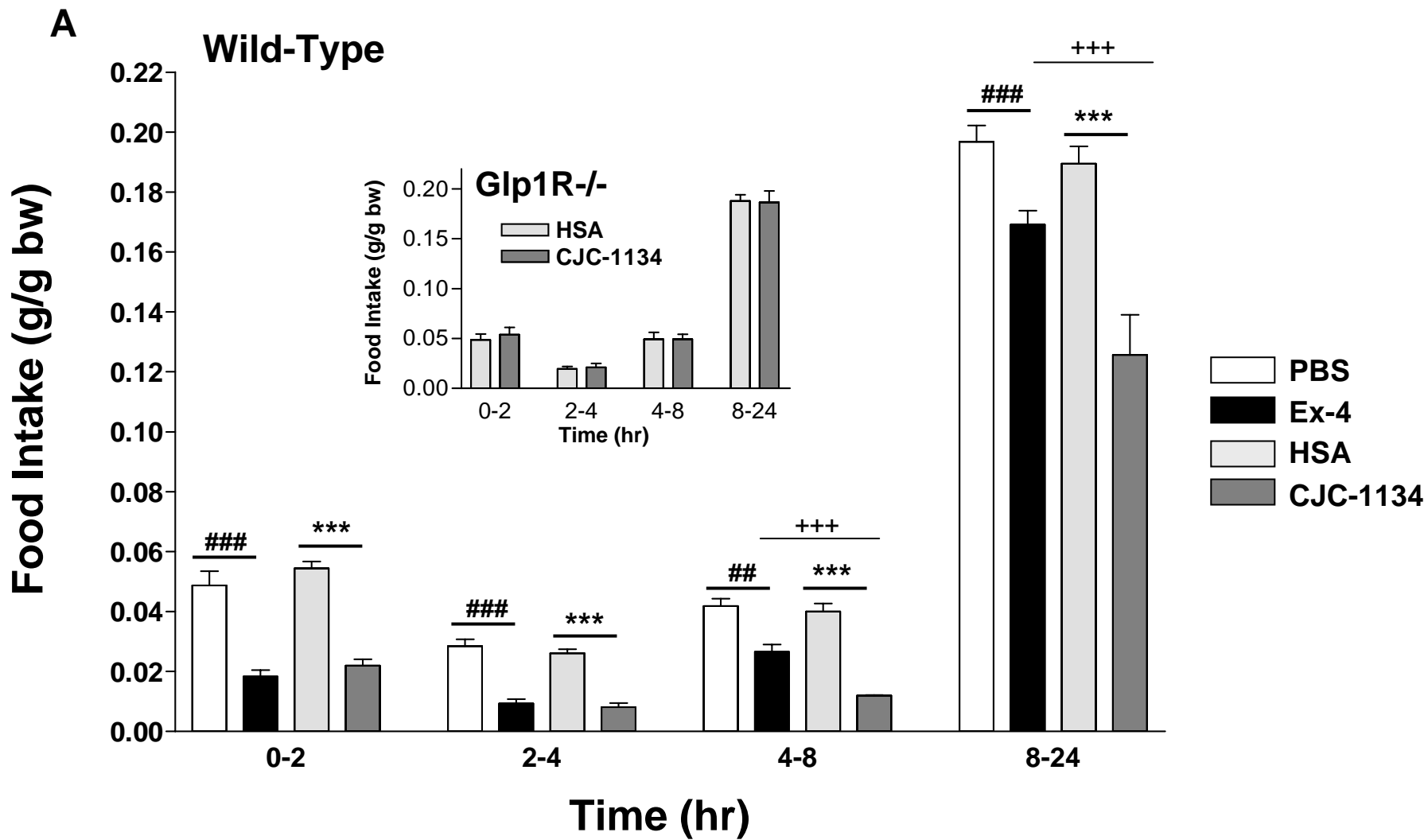
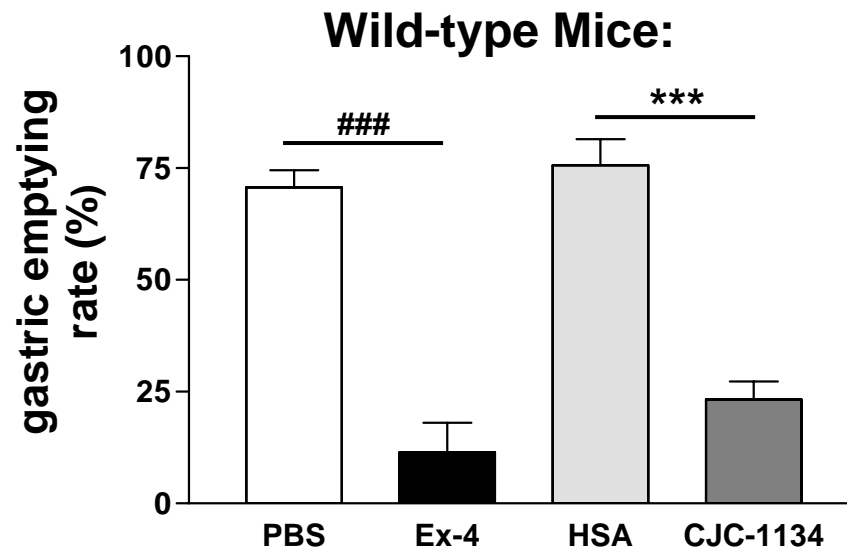
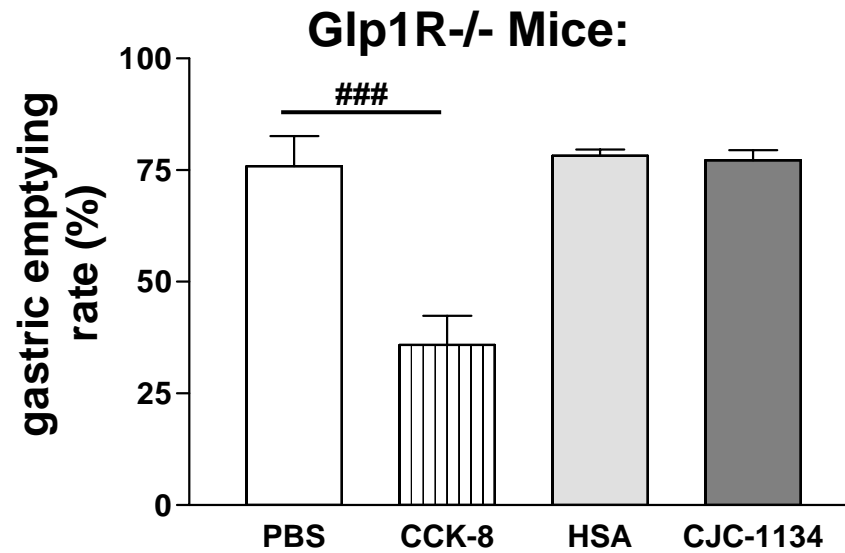


Figure 2

B**C****Figure 2**

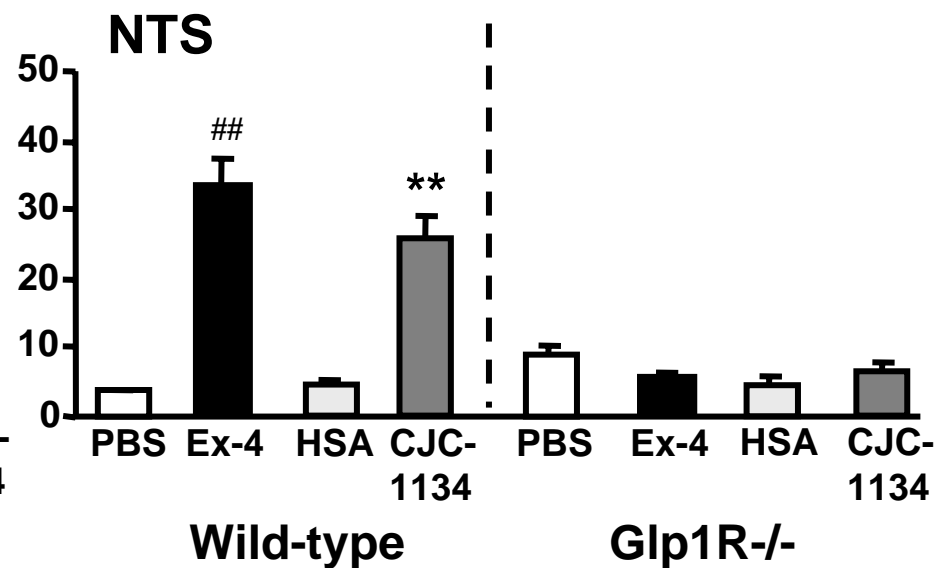
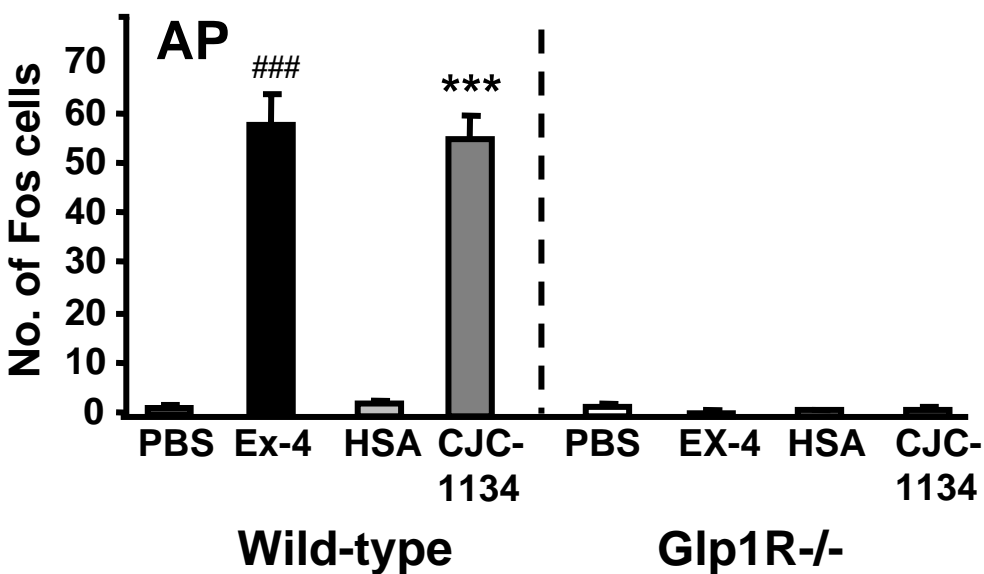
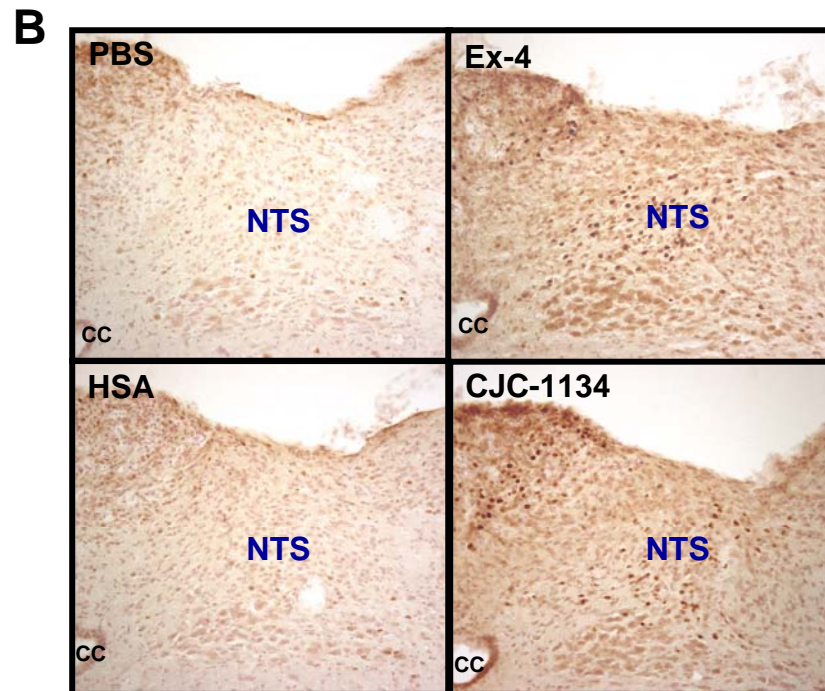
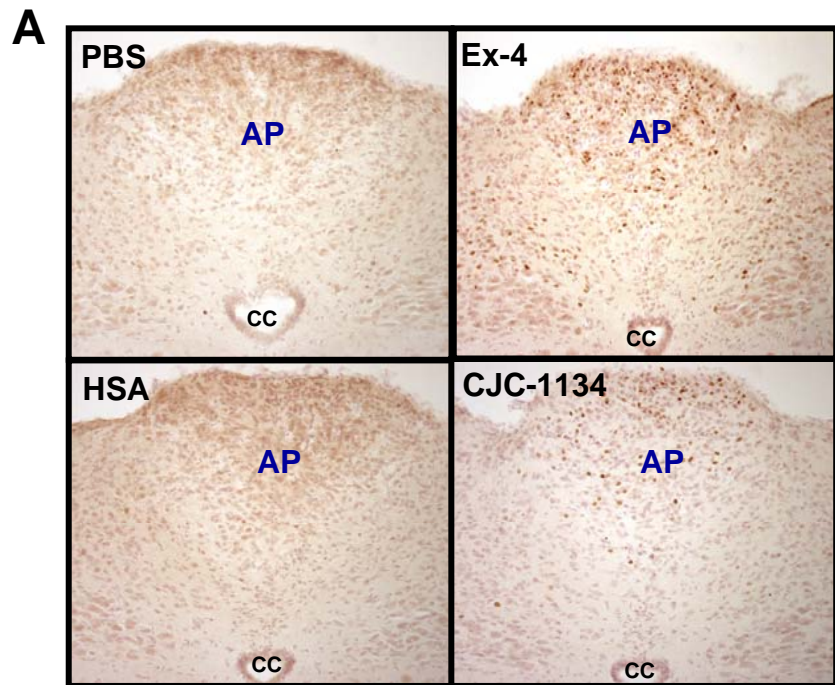


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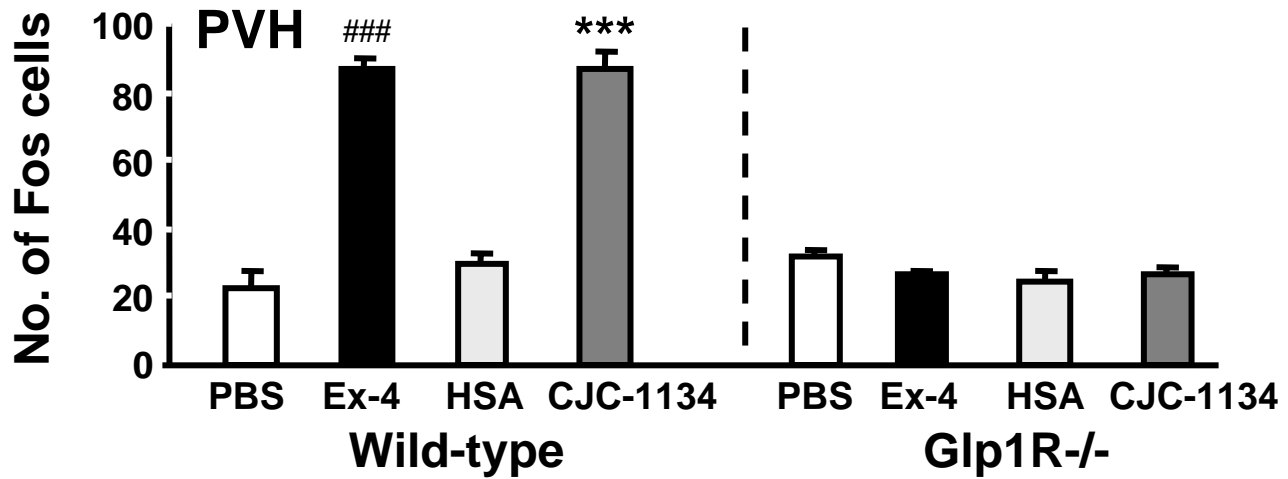
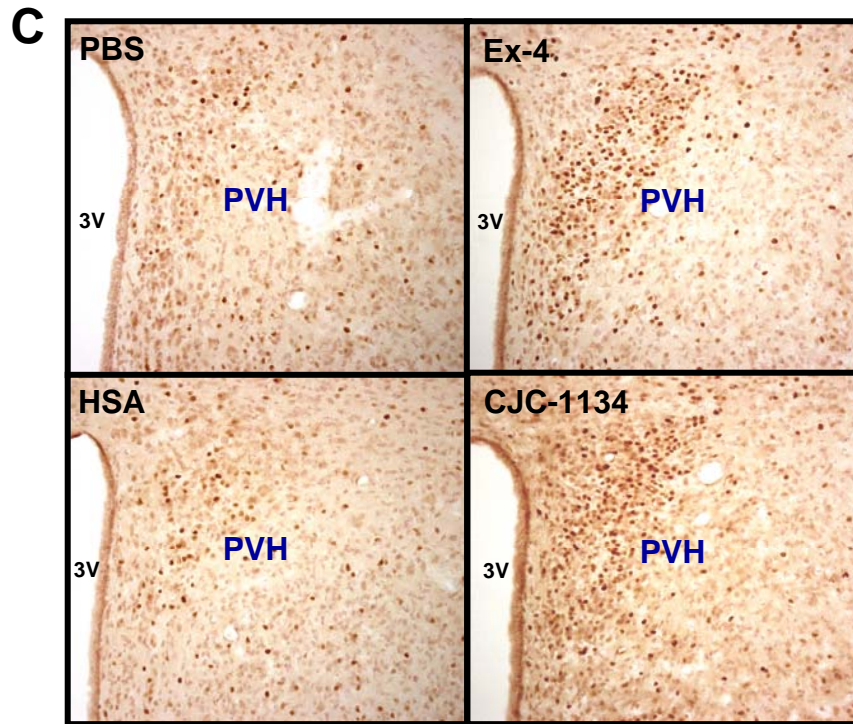


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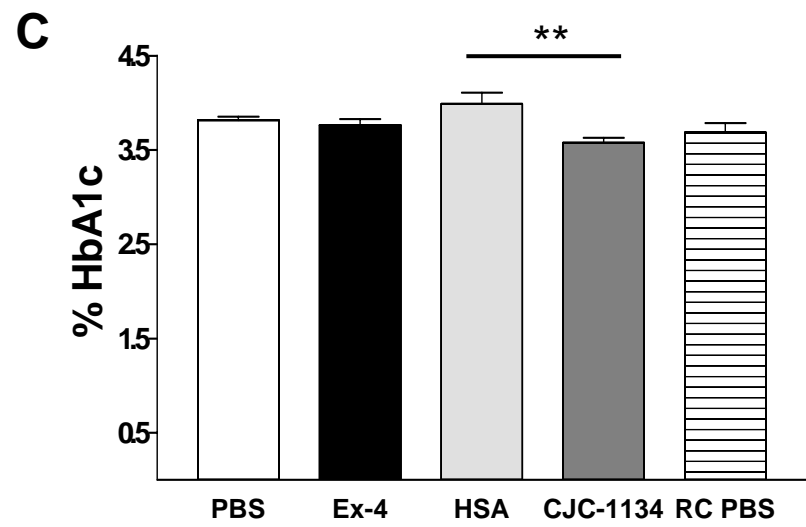
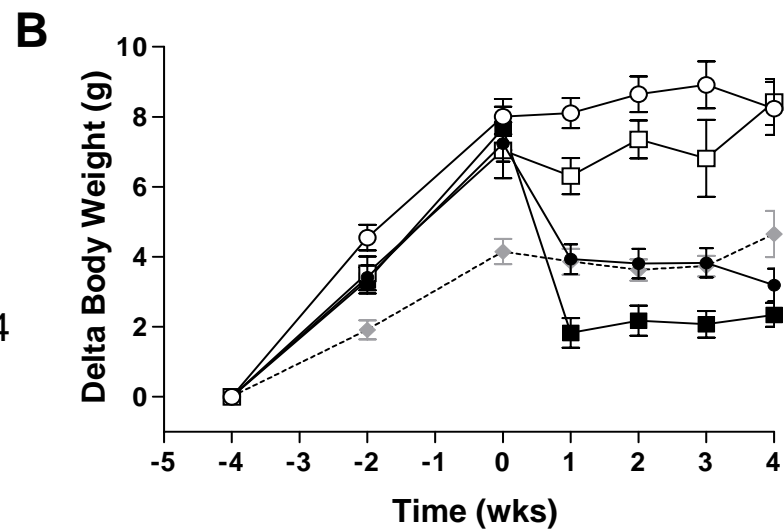
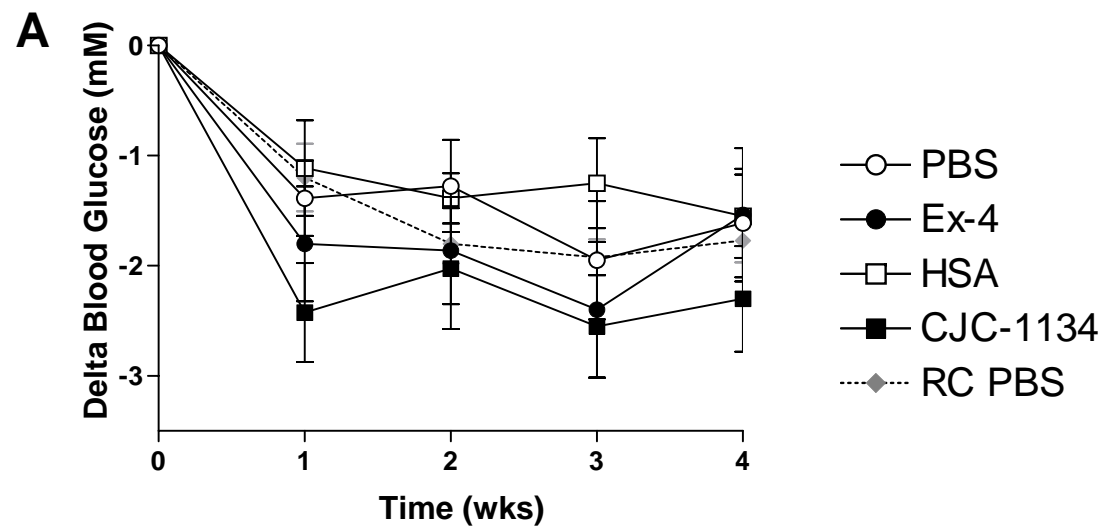


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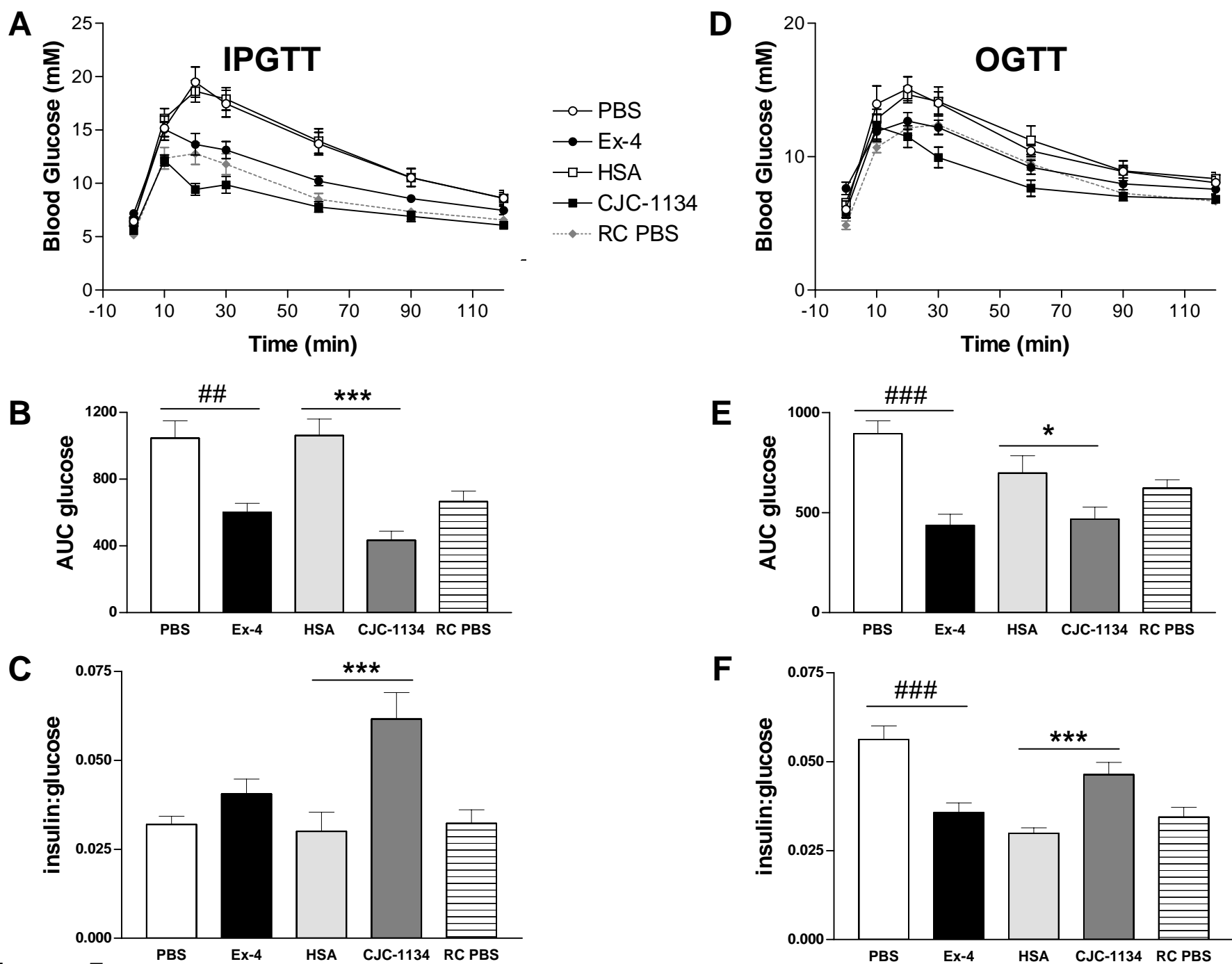


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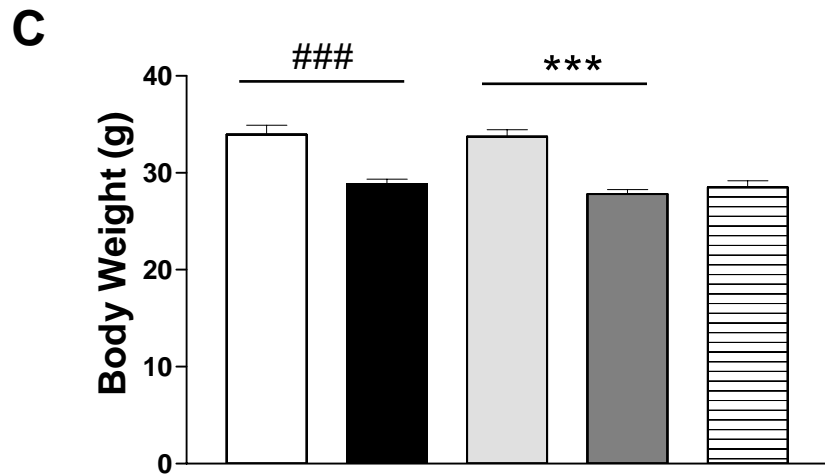
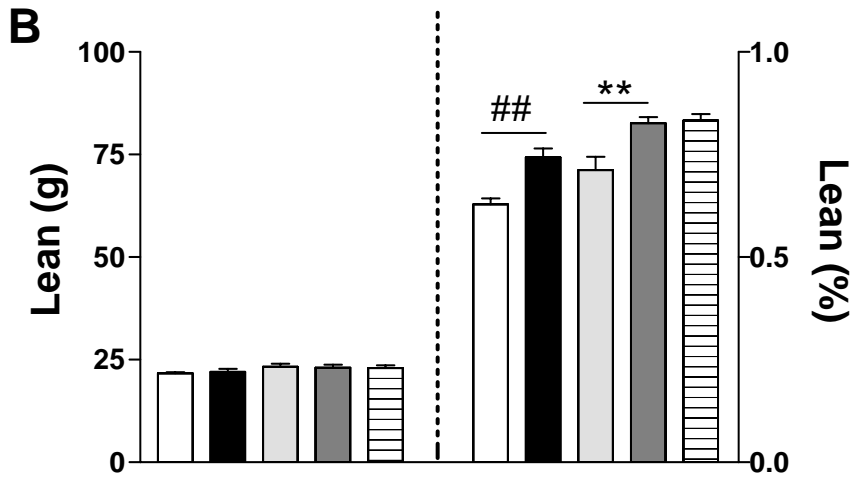
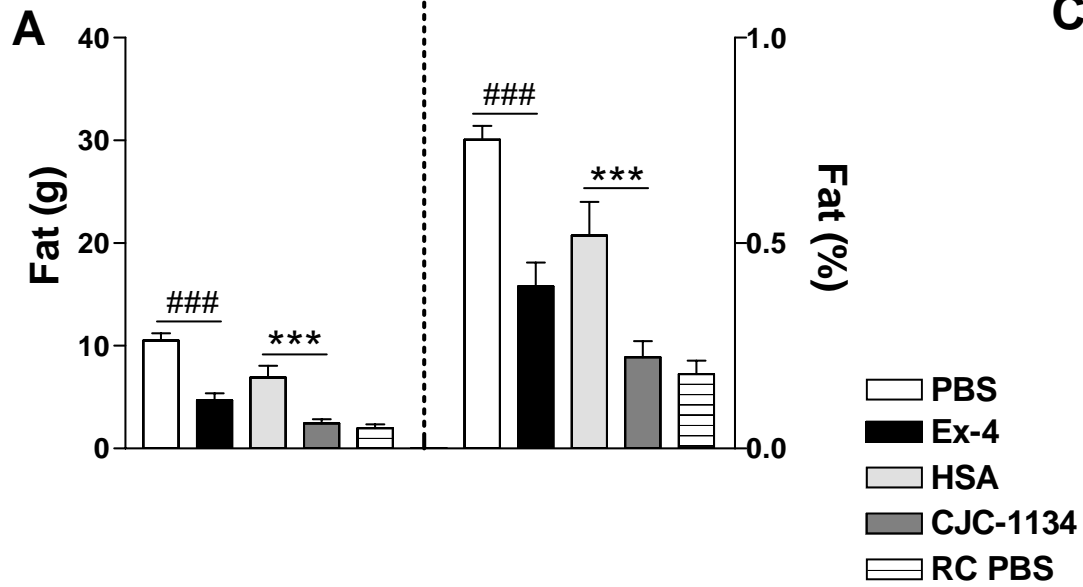


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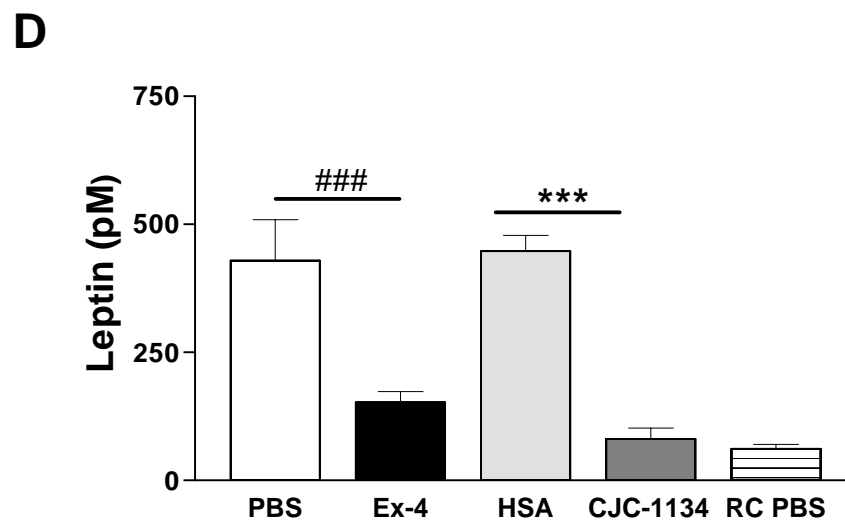
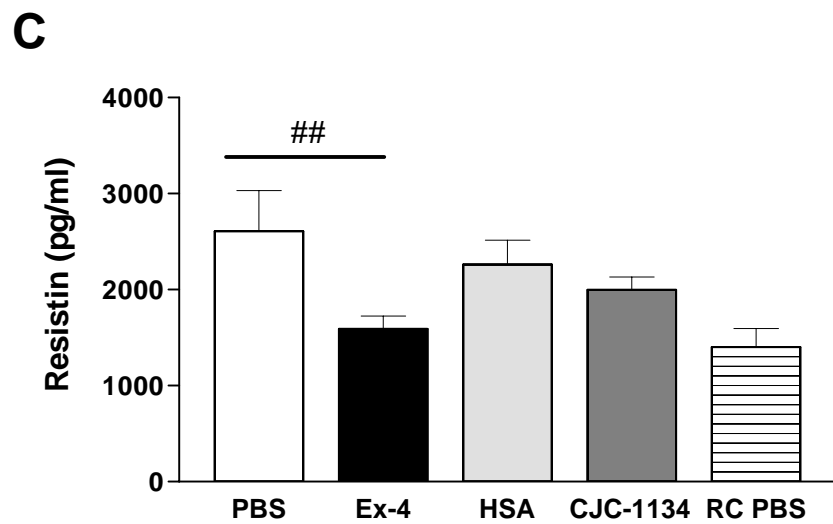
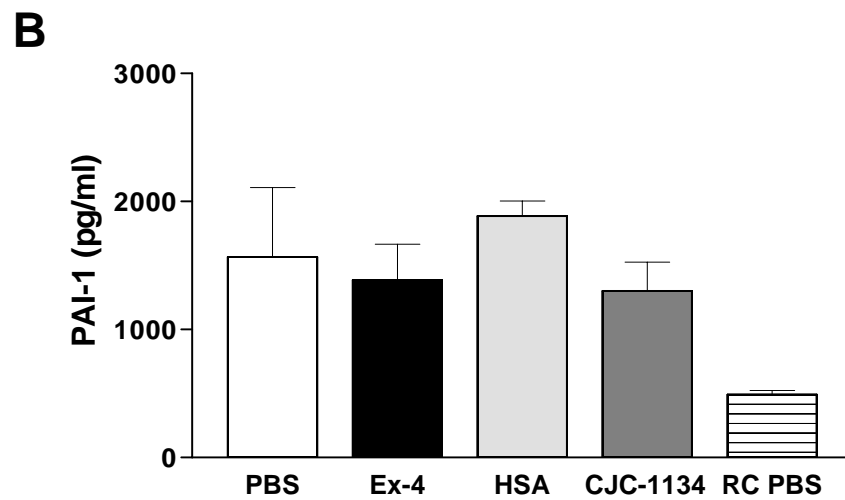
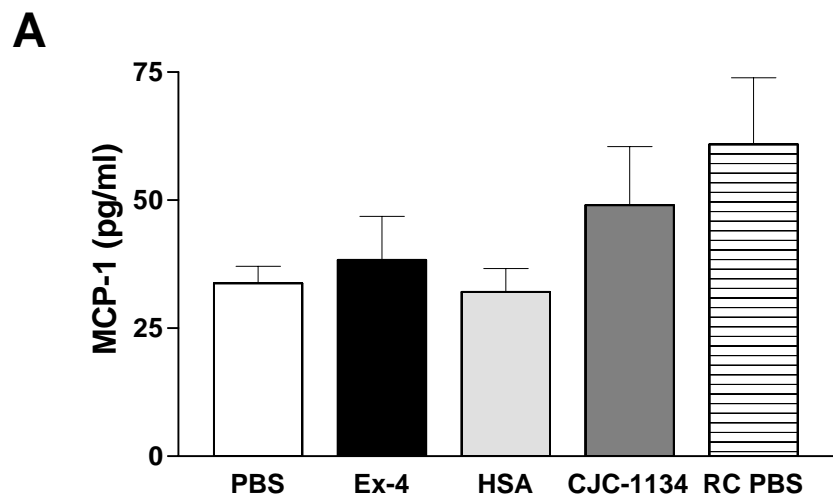


Figure 7

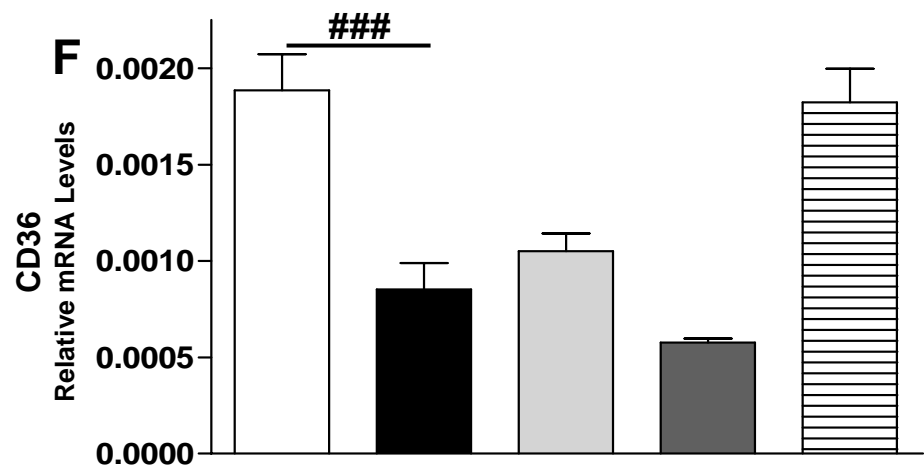
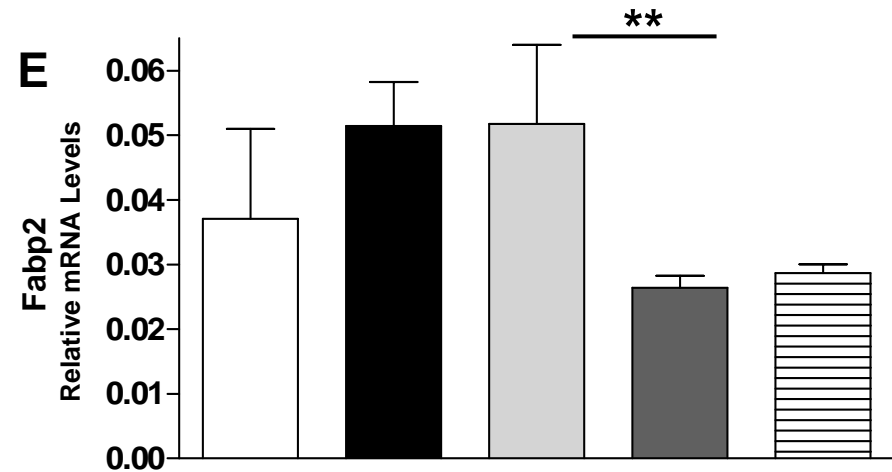
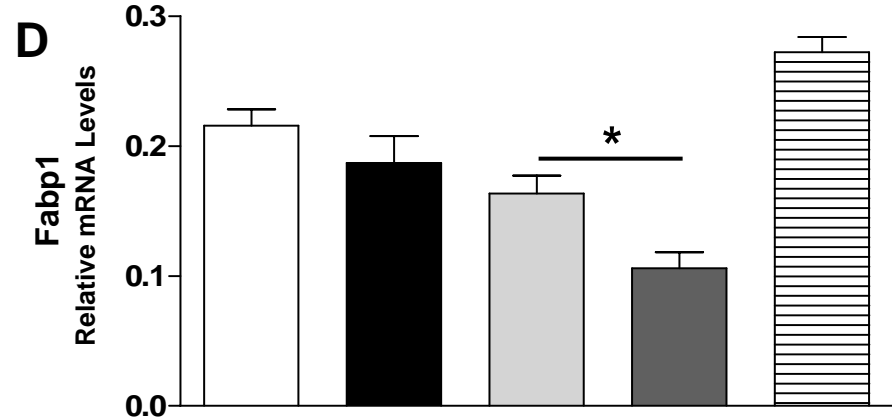
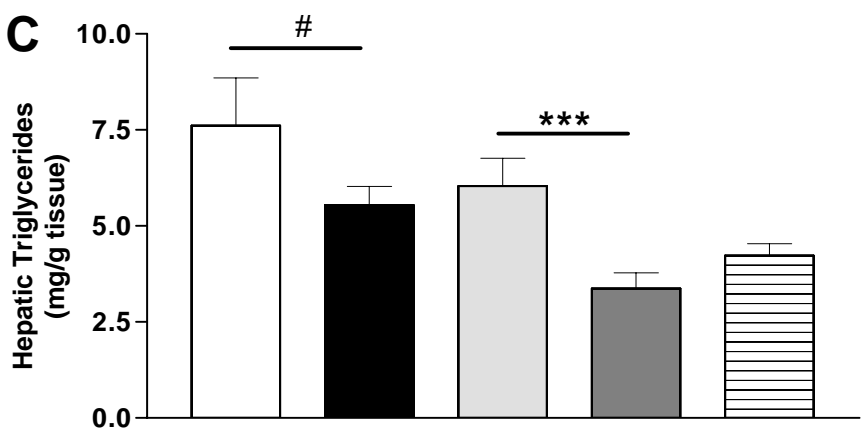
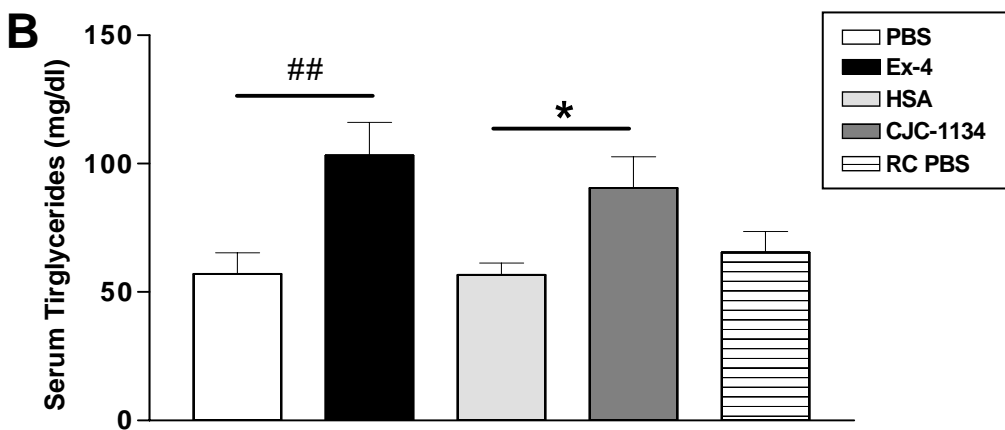
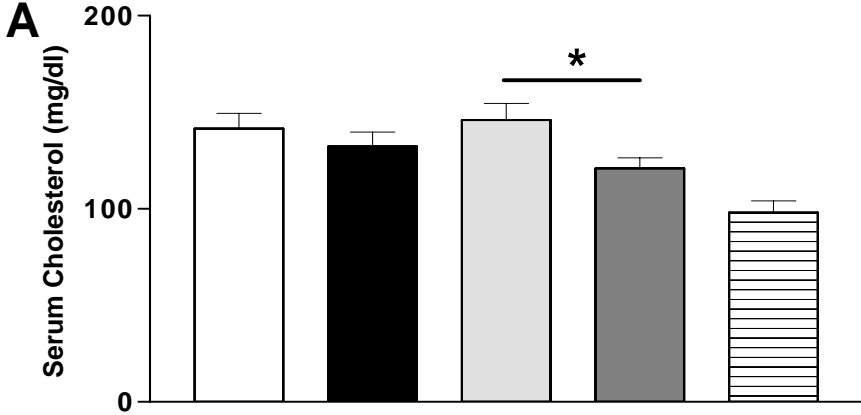
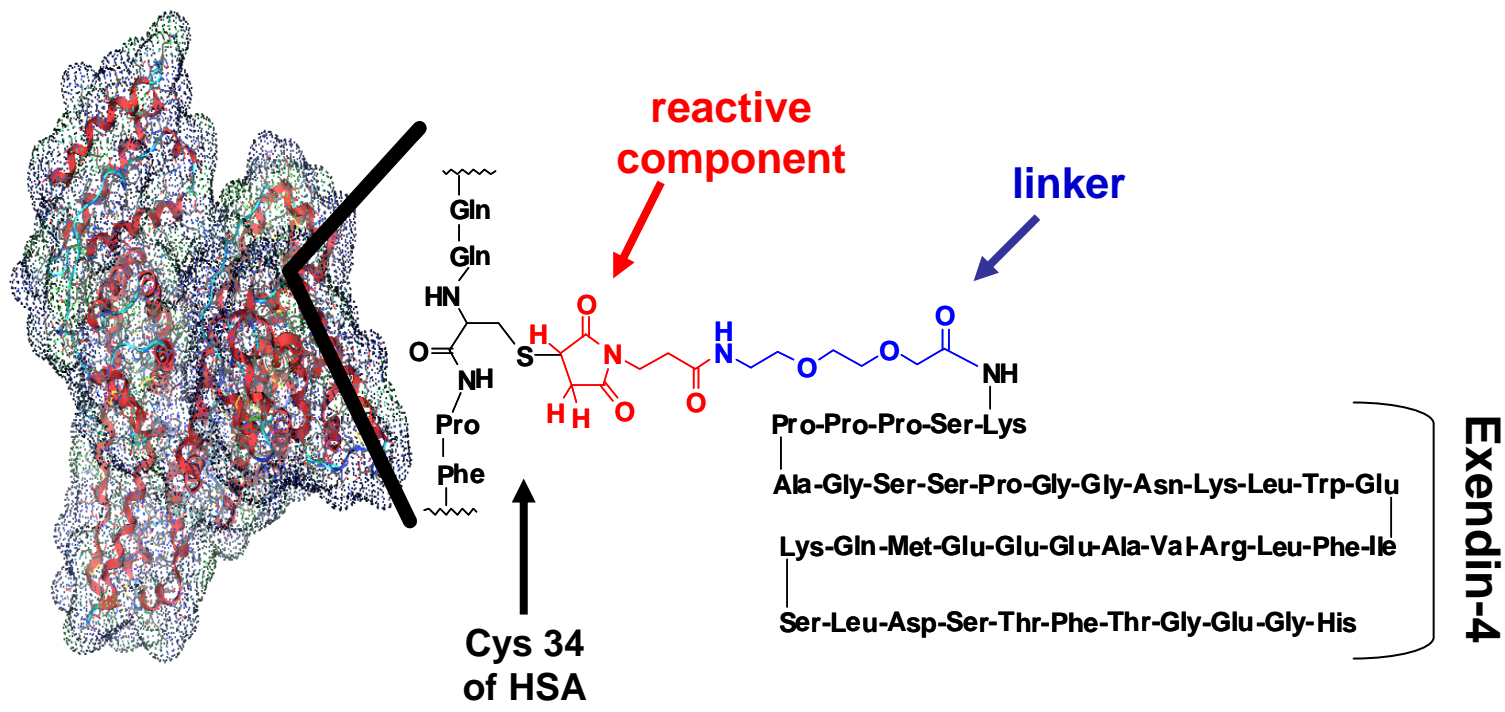
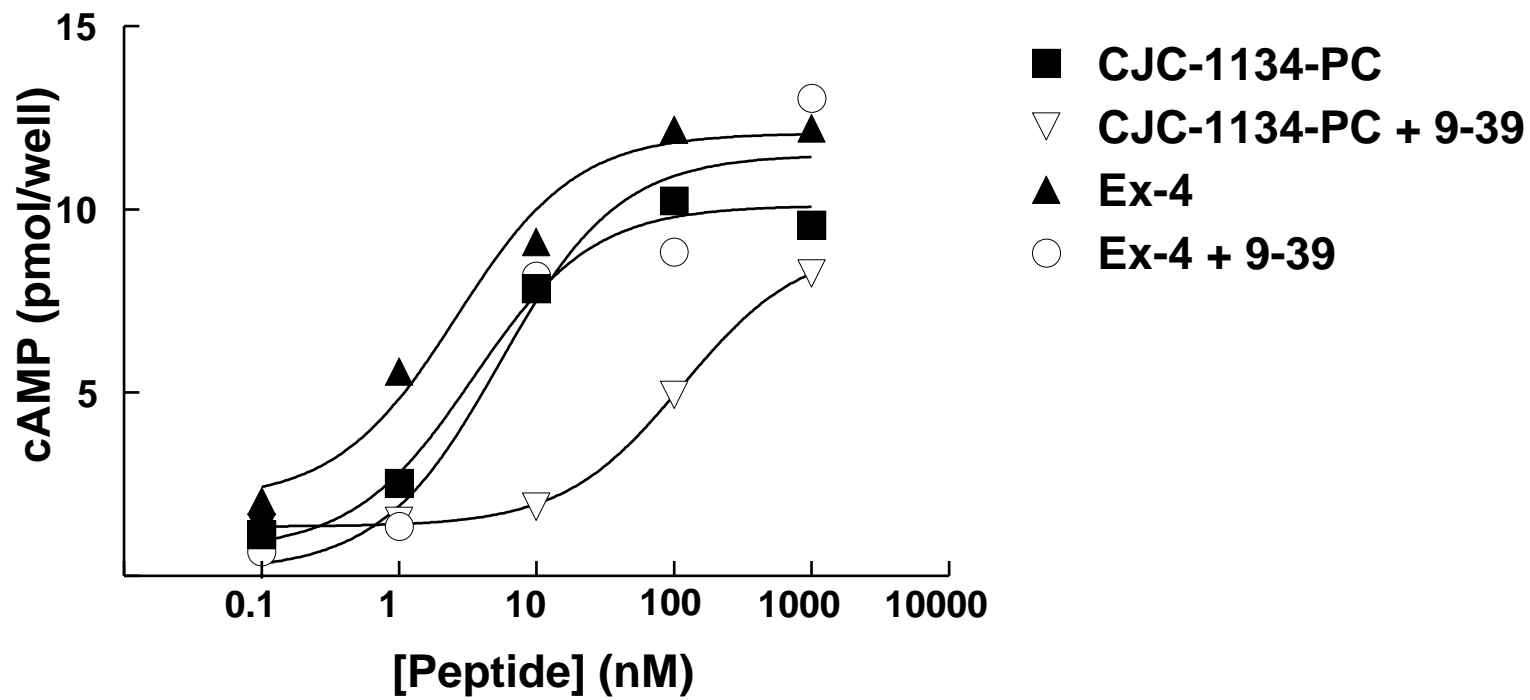


Figure 8

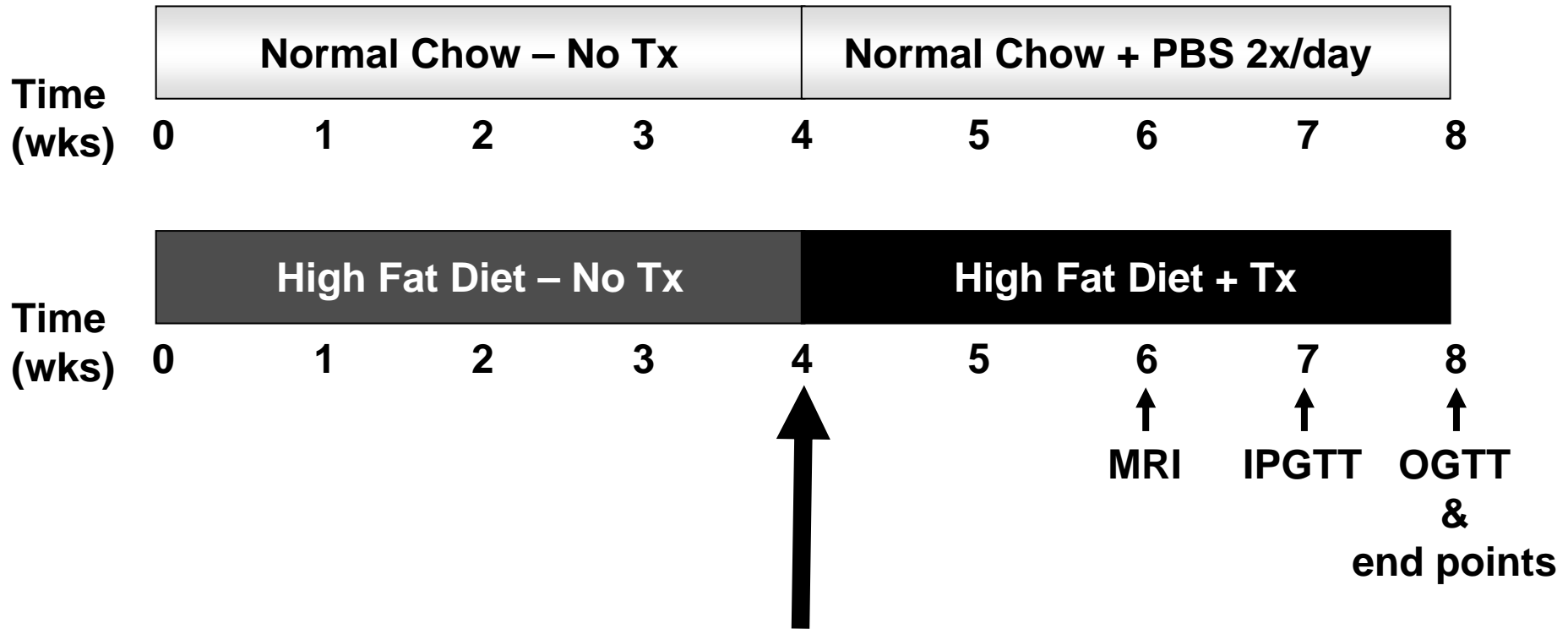


Supplementary Figure 1

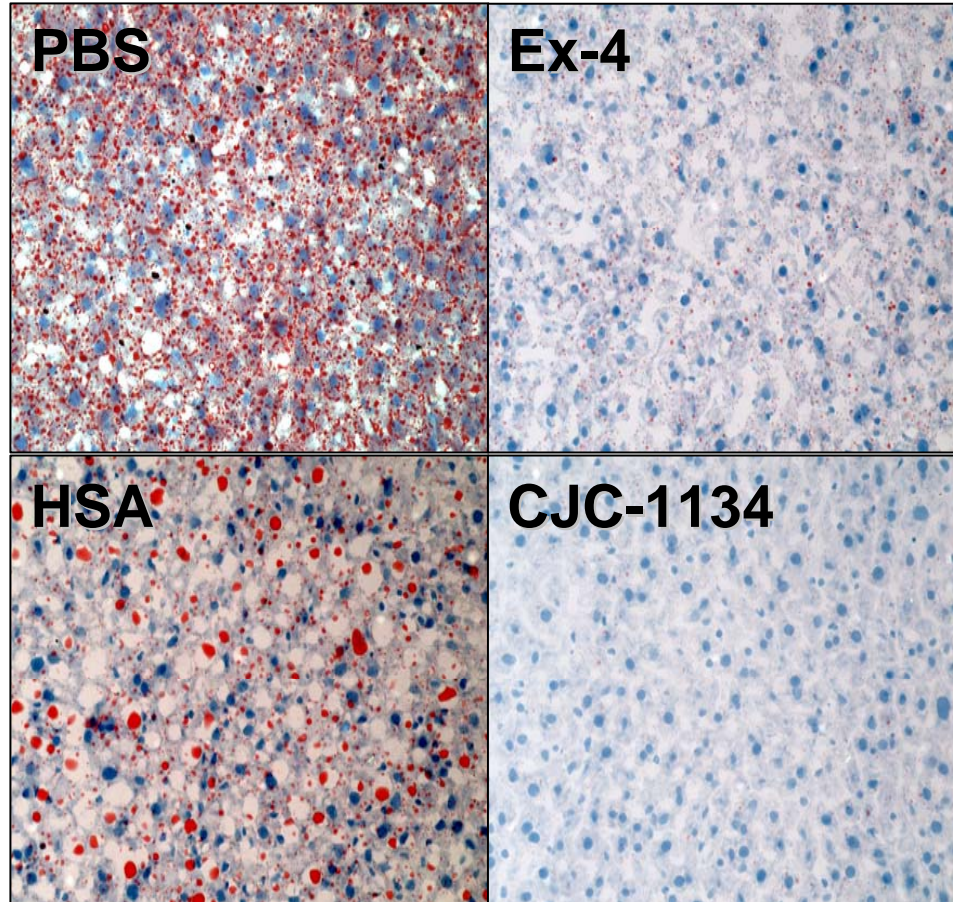


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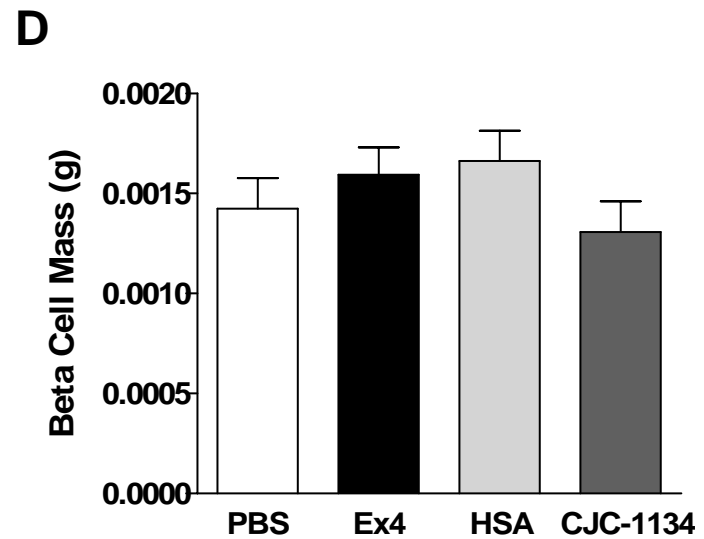
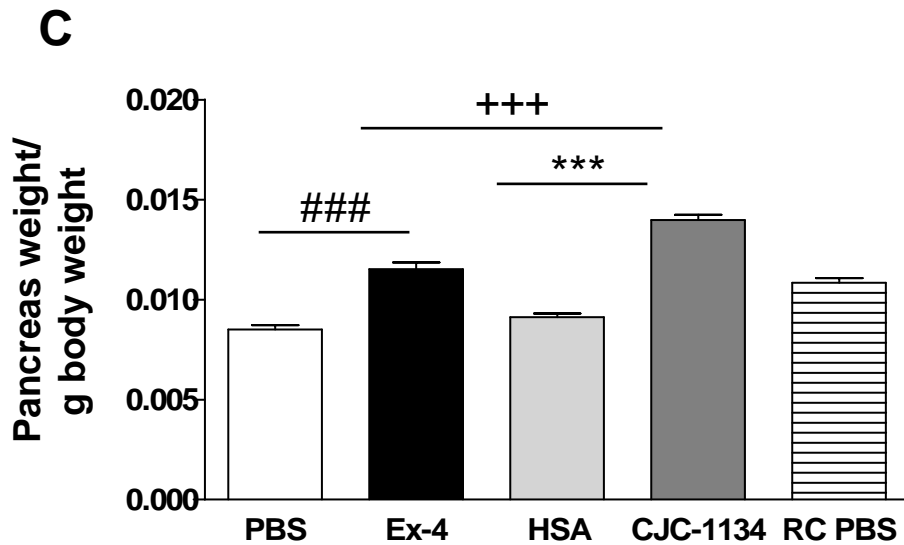
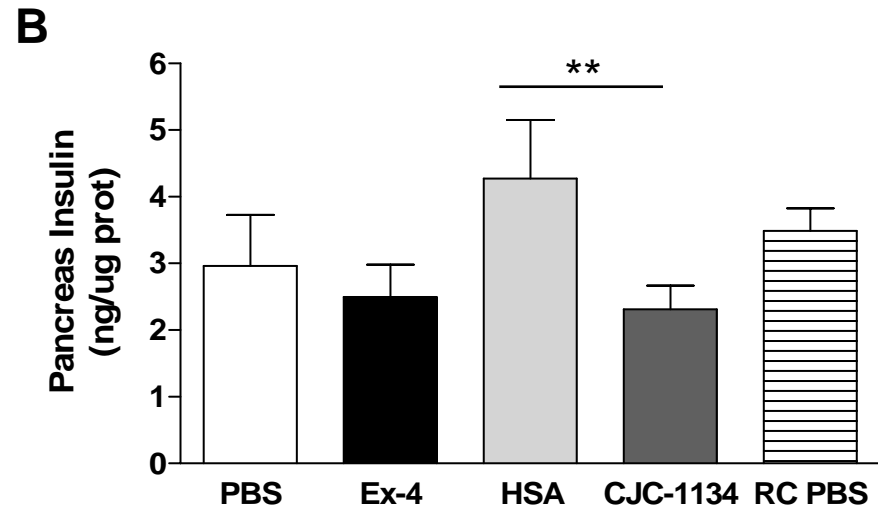
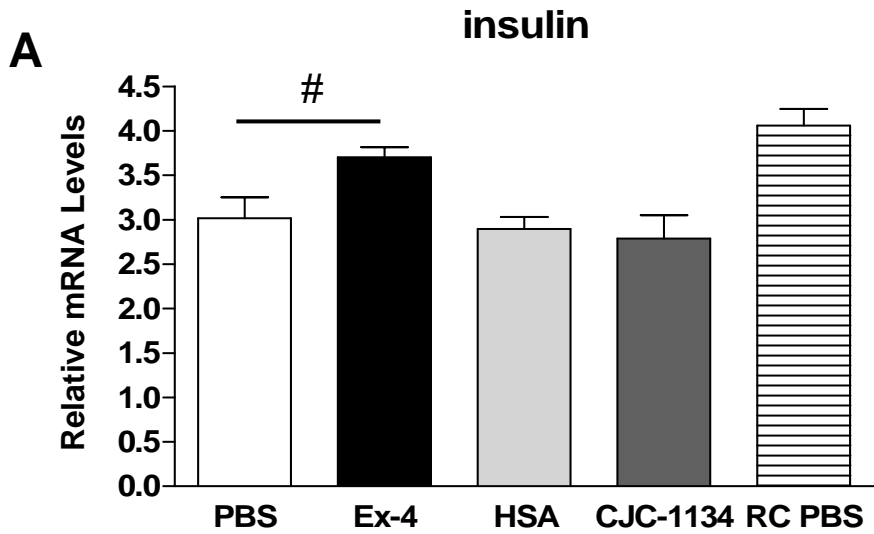
Chronic Studies in wild-type C57BL/6 mice



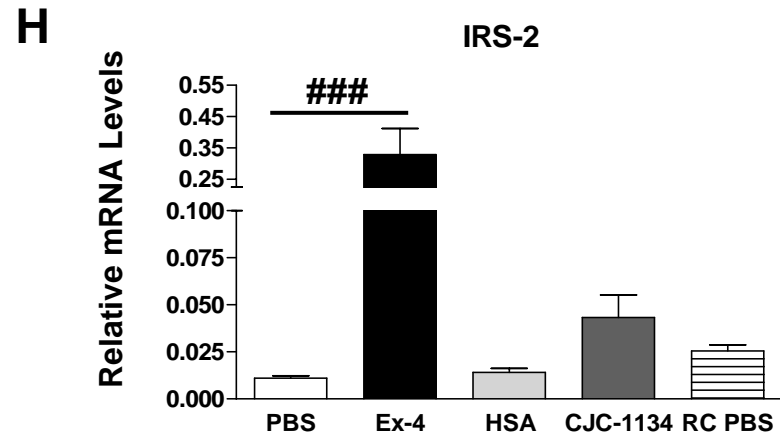
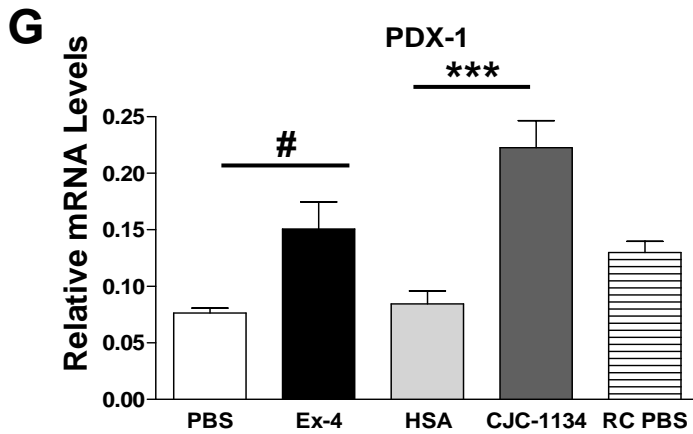
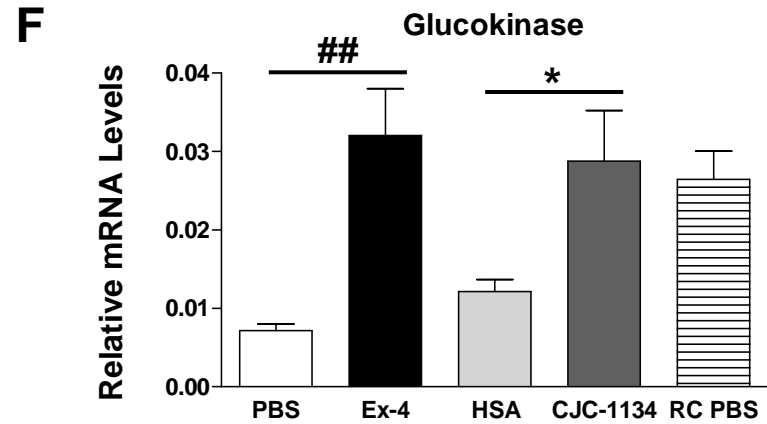
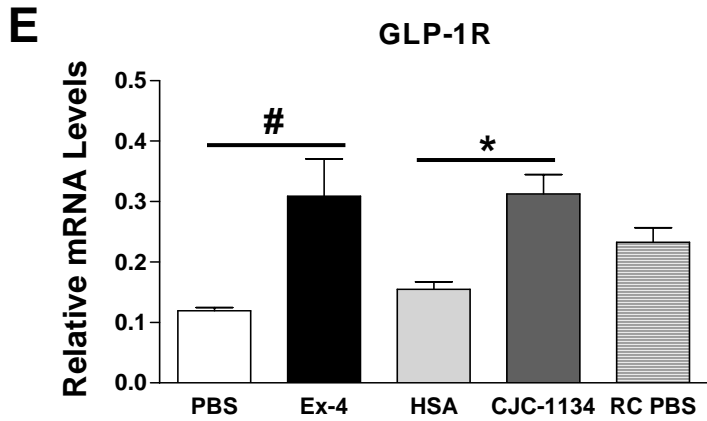
- (i) twice daily (am and pm) PBS
- (ii) twice daily (am and pm) Ex-4 (24 nmol/kg)
- (iii) once daily (pm) HSA (100 nmol/kg)
- (iv) once daily (pm) CJC-1134 (100 nmol/kg)



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 5