"The Hypothalamic Glucagon-Like Peptide-1 (GLP-1) Receptor (GLP-1R) is Sufficient but Not Necessary for the Regulation of Energy Balance and Glucose Homeostasis in Mice"

Running title: Brain GLP-1R regulates nutrient homeostasis

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Word Count: 3989

Figures: 8

Diabetes Publish Ahead of Print, published online December 1, 2016

Abstract

Pharmacological activation of the hypothalamic glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) promotes weight loss and improves glucose tolerance. This demonstrates that the hypothalamic GLP-1R is sufficient but does not show whether it is necessary for the effects of exogenous GLP-1R agonists (GLP-1RA) or endogenous GLP-1 on these parameters. To address this, we crossed mice harboring floxed *Glp1r* alleles to mice expressing Nkx2.1-Cre to knock down *Glp1r* expression throughout the hypothalamus (GLP-1RKD$^{\Delta Nkx2.1cre}$). We also generated mice lacking *Glp1r* expression specifically in two GLP-1RA-responsive hypothalamic feeding nuclei/cell types, the paraventricular nucleus (GLP-1RKD$^{\Delta Sim1cre}$) and proopiomelanocortin neurons (GLP-1RKD$^{\Delta POMCcre}$). Chow -fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice exhibited increased food intake and energy expenditure with no net effect on body weight. When fed a high fat diet (HFD), these mice exhibited normal food intake but elevated energy expenditure, yielding reduced weight gain. None of these phenotypes were observed in GLP-1RKD$^{\Delta Sim1cre}$ and GLP-1RKD$^{\Delta POMCcre}$ mice. The acute anorectic and glucose tolerance effects of peripherally-dosed GLP-1RA exendin-4 and liraglutide were preserved in all mouse lines. Chronic liraglutide treatment reduced body weight in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice, but this effect was attenuated upon HFD feeding. In sum, classical homeostatic control regions are sufficient but not individually necessary for the effects of GLP-1RA on nutrient homeostasis.

Glucagon-like peptide-1 (GLP-1) is a gut-secreted peptide that augments glucose-dependent insulin secretion via a pancreatic GLP-1 receptor (GLP-1R) (1). Long-acting GLP-1R agonists (GLP-1RA) are used for treating type 2 diabetes (T2D) (2). GLP-1RA also reduce food intake and body weight primarily by targeting the central nervous system (CNS) (3). Intracerebroventricular (ICV) injection of GLP-1RA reduces food intake (4-8) and body weight (9) in rodents. Furthermore, CNS deletion of the *Glp1r* attenuates the anorectic effect of the GLP-1RA liraglutide (3). Hypothalamic GLP-1R signaling has received particular attention since this region regulates energy balance as well as glucose and lipid metabolism (10; 11). Peripheral administration of GLP-1RA stimulates hypothalamic neuronal activity (7; 12; 13). Injection of GLP-1RA to hypothalamic nuclei suppresses feeding in rats (5; 14-16), and recent evidence suggests that the arcuate nucleus (ARC) mediates the anorectic effects of liraglutide (17). Furthermore, targeting liraglutide to the ventromedial hypothalamic (VMH) nucleus stimulates brown adipose tissue thermogenesis and adipocyte browning, suggesting that hypothalamic GLP-1R signaling also controls body weight via regulation of energy expenditure (EE) in rodents (15).

The studies described above primarily entailed pharmacological activation of the GLP-1R. Although this demonstrates that the hypothalamic GLP-1R is sufficient to modulate energy balance, it does not address whether it is necessary for the effects of endogenous GLP-1 or clinically utilized GLP-1RAs on phenotypes associated with energy balance. ICV delivery of the GLP-1R antagonist exendin(9-39) (Ex9) stimulates food intake in fed rats and blocks the anorectic effects of peripherally-administered GLP-1RA, suggesting that the CNS GLP-1R is necessary for the feeding effects of endogenous and exogenous GLP-1RA (7; 18). However, this does not identify the specific CNS regions that mediate the satiety effects of GLP-1 or therapeutic GLP-1RA. Furthermore, acute pharmacological approaches do not assess the long-term, day-to-day regulation of energy balance by the CNS GLP-1R. To address this, we generated mice lacking hypothalamic

Glp1r expression by crossing mice expressing floxed *Glp1r* alleles with Nkx2.1-Cre mice. Nkx2.1-Cre mice exhibit Cre expression throughout the hypothalamus including in the ARC, paraventricular (PVN), VMH, dorsomedial (DMH) and lateral (LH) nuclei (19). Since the PVN and ARC have been identified as GLP-1RA-responsive hypothalamic nuclei (14; 17), we also generated mice lacking *Glp1r* expression specifically in the PVN and in POMC neurons (ARC *Glp1r* expression is primarily in POMC neurons (14)) by crossing floxed *Glp1r* mice with Sim1-Cre and POMC-Cre mice, respectively (20; 21). Using these models, we tested the hypothesis that disruption of hypothalamic *Glp1r* expression dysregulates energy balance and that the anorectic effects of peripherally-administered GLP-1RA require the hypothalamic GLP-1R. Since pharmacological studies suggest a role for the hypothalamic GLP-1R in the maintenance of glucose homeostasis (14), we also tested the hypothesis that disruption of hypothalamic *Glp1r* expression impairs glucose tolerance.

We demonstrate that knockdown of the hypothalamic *Glp1r* has no overall effects on net energy balance, glucose homeostasis or the response to peripherally-administered GLP-1RA. These findings highlight the complexity of CNS GLP-1R-mediated signals modulating feeding behavior and glucose handling and indirectly support a role for extrahypothalamic brain regions in GLP-1R-dependent control of energy homeostasis.

Research Design and Methods

Animals and housing

We generated mouse lines lacking *Glp1r* expression in the hypothalamus (GLP-1RKD$^{\Delta Nkx2.1cre}$), PVN (GLP-1RKD$^{\Delta Sim1cre}$) and POMC neurons (GLP-1RKD$^{\Delta POMCcre}$) by breeding floxed *Glp1r* mice (GLP-1R$^{f/f}$), generated as described previously (22), with Nk2 homeobox 1 (Nkx2.1)-Cre, single-minded homolog 1 (Sim1)-Cre, and POMC-Cre mice (Jackson Laboratories, Bar Harbor, ME), respectively (19-21). Experiments were performed in male mice. Chow diet (Harlan Teklad #2016) studies were performed in 3-4-month-old animals. Following energy balance assessment, mice were placed on a 60% high fat diet (HFD) (D12492, Research Diets, New Brunswick, NJ) for 12 weeks, such that HFD-fed studies were performed in 7-8-month-old animals. Animals were maintained on a 12:12-h light:dark cycle in facilities at Sanford Burnham Prebys Medical Discovery Institute at Lake Nona (SBP) or the University of Cincinnati (UC). All protocols were approved by the SBP and UC Institutional Animal Care and Use Committees.

Cannula implantation

Cannulae were implanted as previously described (4). Guide cannulae (Plastics One, Roanoke, VA) targeted the PVN (0.7mm caudal, 0.3mm from midline, 5.0mm ventral), ARC (2.3mm caudal, 1.1mm from midline, 5.6mm ventral, 10º angle), or cortex (0.46mm caudal, 2.0mm from midline, 1.75mm ventral). Verification of proper coordinates was made histologically following injection of 1% Evans Blue dye. Mice recovered for 7 days before experimentation.

Validation of Glp1r knockdown

Glp1r expression was assessed in GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1R$^{f/f}$ and whole body GLP-1R knockout mice (GLP-1RKO) mice by qRT-PCR. Total RNA was extracted from the hypothalamus using a column-based method (Zymo Research, Irvine, CA) and converted to cDNA (High Capacity RNA to cDNA, Applied Biosystems, Foster City, CA). *Glp1r* and ribosomal protein L32 mRNA transcript levels were determined using gene-specific probes in accordance with manufacturer's instructions using a Step One Plus qPCR instrument (Applied Biosystems). qPCR primers (5'→3') were as follows: *Glp1r*, F:GATGCTGCCCTCAAGTGGAT, R:ATGAGCAGGAACACCAGTCG; L32, F:ACATTTGCCCTGAATAGTGGT, R:ATCCTCTTGCCCTGATCCTT. qPCR was also performed in whole brain homogenates excluding the hypothalamus. Gene expression levels were calculated as relative quantification using the $2^{-\Delta\Delta CT}$ method.

Glp1r knockdown in the PVN and ARC of GLP-1RKD$^{\Delta Sim1cre}$ and GLP-1RKD$^{\Delta POMCcre}$ mice was confirmed by RNA *in situ* hybridization. Whole brains were harvested, and tissue was formalin-fixed (24-h, 10% neutral buffered formalin), paraffin-embedded (FFPE) and sectioned using a microtome. 5µm coronal sections were stained with RNAscope probes (Advanced Cell Diagnostics, Newark, CA) according to manufacturer's instructions. Sections were treated with test (*Glp1r*) and control (positive, peptidylprolyl isomerase B; negative, dihydrodipicolinate reductase) RNA probes using a single-plex chromogenic-RED RNAscope 2.5 assay kit on a Bond RX automated ISH slide staining system (Leica Biosystems, Buffalo Grove, IL). The automated protocol included heat retrieval at 88ºC for 15-min and protease retrieval at room temperature for 15-min. Stained slides were coverslipped and scanned using an Aperio ScanScope XT instrument (Leica Biosystems).

Body composition analysis and assessment of energy balance

Lean, fat and fluid mass were measured in 5-h-fasted mice using a LF90II-TD NMR (Bruker, Billerica, MA). Body weights were recorded weekly over the 12-wk HFD feeding period. Energy balance was assessed using a Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH). Animals were acclimated to the CLAMS 24-h prior to the start of experimentation. 48-h food intake, water intake, locomotor activity, EE and respiratory exchange ratio were measured at 15-min intervals as previously described (4).

Food intake response to centrally- and peripherally-administered GLP-1RA

Following cannulation, recovery and acclimation, 18-h food intake was measured after PVN- or ARC-targeted delivery of 0.0025, 0.005 or 0.025µg/100nL GLP-1 or exendin-4 (Ex4,R&D Systems, Minneapolis, MN) or artificial cerebrospinal fluid (ACSF, 100nL bilateral) vehicle (Harvard Apparatus, Holliston, MA) in 5-h-fasted mice just prior to the onset of the dark cycle at 18:00. For peripheral administration, 16-h food intake was measured in mice treated with Ex4 (3µg/kg BW, i.p.), liraglutide (200µg/kg BW, s.c., Novo Nordisk, Copenhagen, Denmark) or saline vehicle. Peripheral doses were chosen based on previously demonstrated anorectic efficacy (3; 23).

Glucose tolerance tests (GTTs)

GTTs were performed on 5-h-fasted mice. In chow-fed mice, 2g/kg BW glucose was administered orally or i.p. In HFD-fed animals, 1g/kg BW glucose was administered orally. GTTs were also performed in animals that received PVN- or ARC-targeted injections of Ex4 (0.025µg/100nL, bilateral) concurrent with an i.p. glucose dose at t=0. In additional experiments, GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta Sim1cre}$ and GLP-1R$^{f/f}$ mice were dosed with the GLP-1R antagonist Ex9 (50µg, i.p., American Peptide) or liraglutide (400µg/kg, s.c.) 15-min (Ex9 studies) or 120-min

(liraglutide studies) prior to an i.p. GTT. Animals were 4-h-fasted, and glucose was administered at a fixed dose (200µl 25% dextrose, Ex9 studies) or at 2g/kg BW for liraglutide studies. Blood glucose measurements were made at the indicated time points. Glucose tolerance was calculated as area under the curve (AUC) above baseline.

Body weight response to peripheral administration of liraglutide

Liraglutide (200ug/kg BW s.c., BID as described by Secher *et al* (17)) or isotonic saline was administered for 14 days in chow and HFD-fed mice, and body weight was measured daily. Body weight was also measured daily during a 7-day recovery period, over which animals received no injection.

Statistical analyses

Data are presented as mean±SEM. Differences between groups were determined by one-way ANOVA followed by Tukey or Newman-Keuls Multiple Comparison post-hoc tests or by two-tailed *t*-test as appropriate. Statistical significance was set to $p < 0.05$.

Results

Targeted delivery of GLP-1 or Ex4 to the PVN and ARC dose-dependently suppresses food intake.

Consistent with previous studies in rats (5; 14; 15), targeting GLP-1 to the PVN (Figure 1A) but not the ARC (Figure 1B) significantly reduced food intake in mice. Targeting Ex4 to the PVN (Figure 1C) or ARC (Figure 1D) potently suppressed food intake, particularly during the first 12 hours post-administration. These findings demonstrate that pharmacological activation of the PVN or ARC GLP-1R reduces food intake in mice, although the magnitude and duration of the response depend on the GLP-1RA administered and region targeted.

Gene expression of the hypothalamic Glp1r is reduced by Cre-lox recombination.

To test whether the hypothalamic GLP-1R is necessary for chronic maintenance of nutrient homeostasis, we bred floxed *Glp1r* mice with a line expressing Cre recombinase in multiple hypothalamic regions, Nkx2.1-Cre mice (19), to generate hypothalamic *Glp1r* knockout (GLP-1RKD$^{\Delta Nkx2.1cre}$) mice. *Glp1r* mRNA levels were significantly reduced in hypothalami from GLP-1RKD$^{\Delta Nkx2.1cre}$ mice, although not to the same extent as in GLP-1RKO mice (Figure 2A). *Glp1r* mRNA levels were unaffected in the cortex (non-hypothalamic control) of GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Figure 2A). Since the PVN and ARC have been shown to mediate the anorectic effects of pharmacological GLP-1RA, we also generated mice lacking *Glp1r* expression in the PVN (GLP-1RKD$^{\Delta Sim1cre}$ mice) and POMC neurons (GLP-1RKD$^{\Delta POMCcre}$ mice). Selective deletion in POMC neurons was chosen because the *Glp1r* is expressed in anorexigenic POMC but not in orexigenic NPY/AgRP neurons of the ARC (14). Knockdown of the *Glp1r* in GLP-1RKD$^{\Delta Sim1cre}$ mice was

restricted to the PVN (Figure 2B), and knockdown of the *Glp1r* in GLP-1RKD$^{\Delta POMCcre}$ mice was restricted to the ARC (Figure 2C).

Disruption of Glp1r expression in Nkx2.1 neurons elevates food intake and energy expenditure with no alterations in body weight or composition in chow-fed mice.

Total, lean and fat mass were unaltered in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Figure 3A) although there was a tendency (p=0.07) for reduced fat mass relative to total body mass as shown by analysis of covariance (ANCOVA) (Supplemental Table 1). Disruption of *Glp1r* expression in chow-fed GLP-1RKD$^{\Delta POMCcre}$ (Figure 3B) and GLP-1RKD$^{\Delta Sim1cre}$ (Figure 3C) mice also had no effect on body weight or composition. GLP-1RKD$^{\Delta Nkx2.1cre}$ mice displayed significantly elevated 48-h food (Figure 3D) and water (Supplemental Figure 1A) intake. Basal 48-h EE was also elevated in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Figure 3G). This was not due to an appreciable increase in locomotor activity (Supplemental Figure 1G), nor was there any change in the respiratory exchange ratio (RER) (Supplemental Figure 1D). Increased EE in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice occurred independently from food intake or total body mass (Supplemental Table 1). These results indicate that the hypothalamic *Glp1r* plays a role in the regulation of basal food intake and EE.

Targeted disruption of Glp1r expression in POMC neurons and the PVN elicits minor effects on energy balance in chow-fed mice.

We next determined whether the elevated food intake and energy expenditure in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice was due to loss of *Glp1r* expression in POMC neurons or the PVN. Knockdown of *Glp1r* within POMC neurons conferred no changes in basal food intake (Figure 3E) or EE (Figure 3H). Although knockdown of the GLP-1R specifically within the PVN conferred no changes in basal food intake (Figure 3F), there was a significant decrease in EE (Figure 3I). There

were no significant effects on water intake, RER or locomotor activity in GLP-1RKD$^{\Delta POMCcre}$ or GLP-1RKD$^{\Delta Sim1cre}$ mice (Supplemental Figure 1).

Glp1r knockdown in Nkx2.1-expressing neurons protects mice from HFD-induced weight gain and increased fat mass, and this phenotype is associated with elevated energy expenditure.

We next determined whether the body composition and energy balance phenotypes observed in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice persist after HFD feeding. GLP-1RKD$^{\Delta Nkx2.1cre}$ mice displayed a significantly reduced HFD-induced weight gain and fat mass (Figures 4A and 4D). In contrast to chow-fed animals, HFD-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice did not exhibit increased 48-h food intake (Figure 4G). Despite this, 48-h water intake was significantly elevated in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Supplemental Figure 2A). 48-h EE remained significantly elevated in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Figure 4J), and this was independent of the difference in total body weight compared to controls (Supplemental Table 1). Elevated EE was not due to an appreciable difference in locomotor activity (Supplemental Figure 2G). There was no difference in the RER (Supplemental Figure 2D). Thus, loss of *Glp1r* expression in Nkx2.1-expressing neurons increases EE regardless of diet and independently of effects on food intake.

Disruption of Glp1r expression in POMC neurons and the PVN elicits variable effects on energy balance in HFD-fed mice.

We assessed whether the protection from HFD-induced obesity and increased EE in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice was due to loss of *Glp1r* expression in POMC neurons or the PVN. Contrary to GLP-1RKD$^{\Delta Nkx2.1cre}$ mice, GLP-1RKD$^{\Delta POMCcre}$ mice displayed increased HFD-induced weight gain (Figure 4B). However, there were no significant differences in lean or fat mass between genotypes (Figure 4E). No differences in HFD-induced weight gain or body composition were

observed in GLP-1RKD$^{\Delta Sim1cre}$ mice (Figures 4C and 4F). Additional parameters of energy balance in these mice were unaltered (Figures 4H, 4I, 4K and 4L and Supplemental Figure 2). These findings suggest that the energy balance phenotypes observed in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice are not due to loss of *Glp1r* expression in the PVN or POMC neurons.

Hypothalamic Glp1r knockdown mice exhibit a normal anorectic response to acute, peripheral administration of Ex4.

We next tested the hypothesis that loss of hypothalamic *Glp1r* expression blunts the acute anorectic effect of peripherally-dosed GLP-1RA. Suppression of food intake by Ex4 was equivalent in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta POMCcre}$ and GLP-1RKD$^{\Delta Sim1cre}$ (Figures 5A -5C) compared to their respective controls. Ex4 also suppressed food intake similarly in HFD-fed GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta POMCcre}$ and GLP-1RKD$^{\Delta Sim1cre}$ (Figures 5D-5F) mice compared to controls. These findings show that the GLP-1R in Nkx2.1-expressing neurons and classical hypothalamic feeding centers (i.e., PVN and POMC neurons) is not required for the acute anorectic effects of peripherally-administered GLP-1RA.

Loss of the Glp1r in Nkx2.1-expressing neurons attenuates the body weight-reducing effect of chronic liraglutide treatment only in HFD-fed mice.

Liraglutide does not reduce 24-h food intake in mice with CNS deletion of the GLP-1R (3). We tested the hypothesis that this is due to loss of hypothalamic *Glp1r* expression. Loss of *Glp1r* expression in Nkx2.1-expressing neurons did not affect the acute food intake-suppressive effect of liraglutide (Figure 6A). We then tested whether loss of hypothalamic *Glp1r* expression attenuates the body weight-reducing effects of a chronic dosing regimen of liraglutide. Secher and colleagues (17) recently demonstrated that infusion of the GLP-1R antagonist Ex9 into the ARC partially

attenuates the anorectic effects of a 14-day liraglutide treatment. We show that the body weight-reducing effect of liraglutide was unaffected in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice (Figure 6B). However, loss of hypothalamic *Glp1r* expression attenuated the weight-reducing effect of liraglutide in HFD-fed mice, suggesting that the hypothalamic GLP-1R is required for peripherally-dosed liraglutide to exert its body weight-reducing effect during the metabolic stress of a HFD.

Glucose tolerance is improved by pharmacological activation of the GLP-1R in the PVN and ARC, but loss of Glp1r expression in these brain regions does not impair glucose tolerance.

Brain GLP-1R signaling modulates peripheral glucose production and/or utilization (14; 24-27). We assessed the effect of targeted delivery of Ex4 to specific hypothalamic nuclei on glucose tolerance. An anorectic dose of Ex4 (0.025 μg) targeted to the PVN (Figure 7A) or ARC (Figure 7B), but not the cortex (Figure 7C), improved glucose tolerance.

We then assessed whether loss of *Glp1r* expression in Nkx2.1 neurons, the PVN or POMC neurons impairs glucose handling. Oral (Figure 7D-7F) and i.p. (Figure 7G-7I) glucose tolerance was identical in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta POMCcre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice compared to respective controls. Similarly, oral glucose tolerance was unaffected by the loss of *Glp1r* expression in these brain regions in HFD-fed mice (Figure 7J-7L). Pre-treatment with the GLP-1R antagonist Ex9 impaired glucose tolerance similarly in GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice (Figure 8A). Thus, although pharmacological activation of the hypothalamic GLP-1R improves glucose tolerance, the GLP-1R in this brain region is not necessary for glucose handling.

Liraglutide improves glucose tolerance independently of the hypothalamic GLP-1R.

Neuronal-wide deletion of the GLP-1R does not affect the ability of liraglutide to improve glucose tolerance (3). However, global CNS deletion of the GLP-1R could mask the contributions of specific brain regions to the regulation of glucose tolerance. Pre-treatment with liraglutide improves i.p. glucose tolerance similarly in both GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice (Figure 8B), suggesting that the hypothalamic GLP-1R is not required for the glucoregulatory effect of peripherally-administered liraglutide.

Discussion

GLP-1RA suppress food intake and reduce body weight via food intake-regulatory brain regions including the hypothalamus and brainstem (17; 28). We and others have shown that GLP-1RA targeted to the hindbrain (29) and hypothalamic nuclei including the PVN (30), DMH (5) and ARC reduce food intake. However, these acute pharmacological interventions do not reflect the physiological, long-term regulation of feeding by the brain GLP-1R. The present studies assessed the impact of selectively knocking down hypothalamic *Glp1r* expression on overall energy balance. *Glp1r* expression was disrupted in Nkx2.1-expressing neurons, PVN or POMC neurons to address whether these regions are necessary for the anorectic and glucoregulatory effects of GLP-1RA. We report that loss of *Glp1r* expression in these three regions does not affect energy balance or responsiveness to peripheral administration of GLP-1RA. This is surprising since targeted administration of GLP-1RA to these regions potently reduces food intake and improves glucose tolerance. These findings demonstrate that classical GLP-1RA-responsive hypothalamic neurons are not necessary for the anorectic and glucoregulatory effects of GLP-1RA.

The GLP-1R is expressed in extrahypothalamic food intake-regulatory regions including the central amygdala, hindbrain and area postrema (31). Several studies have also shown that targeting GLP-1RA to reward regions including the ventral tegmental area (VTA) (32), nucleus accumbens (NAc) (33) and lateral parabrachial nucleus (LPBN) (34) suppresses food intake. Thus, CNS GLP-1R signaling could reduce food intake and body weight by decreasing the hedonic value of food (35). Anorectic effects of GLP-1RA may also be secondary to homeostatic stress (36; 37). GLP-1 signaling in the PVN and hindbrain regulates the behavioral, autonomic and neuroendocrine responses to stress by activating both the “fight or flight” response acutely and the hypothalamic-pituitary-adrenal (HPA) axis chronically (36-40). Moreover, the central GLP-1R system is

activated in response to stressful stimuli, which can themselves reduce food intake and elevate blood glucose (37; 41). The GLP-1R is also expressed in peripheral vagal afferent neurons (42), yet the extent to which these neurons relay gut-derived GLP-1R signals to the brain to mediate satiating and glucoregulatory responses remains controversial. Kanoski *et al* demonstrated that the anorectic effects of peripherally-dosed Ex4 and liraglutide involve activation of GLP-1R both on vagal afferents and in the CNS (18), whereas Secher *et al* report that subdiaphragmatic vagal afferent deafferentiation does not impact the food intake- and body weight-lowering effects of peripherally-dosed liraglutide (17).

GLP-1R KD$^{\Delta Nkx2.1cre}$ mice display elevated 48-h food intake and EE. The net effect likely explains the absence of a body weight effect in these mice. Knockdown of the GLP-1R in the PVN or POMC neurons did not recapitulate the increased food intake and EE phenotypes observed in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice, suggesting that alternative *Glp1r*-expressing hypothalamic regions such as the VMH, DMH or LH (31) may be involved in regulating these processes. We then hypothesized that loss of hypothalamic *Glp1r* would exacerbate the metabolic derangements of HFD feeding. On the contrary, GLP-1RKD$^{\Delta Nkx2.1cre}$ mice were protected from HFD-induced weight and fat mass gain. This was not due to reduced food intake. Instead, the elevated EE observed in chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice persisted with HFD feeding, demonstrating that loss of hypothalamic *Glp1r* expression increases EE independently of food intake. This is surprising because ICV or hypothalamic administration of GLP-1RA increases EE (15; 43). Loss of hypothalamic *Glp1r* expression may increase sensitivity to GLP-1 in other brain regions, thus, elevating EE. However, whole body *Glp1r* KO mice also exhibit increased EE (44). Paradoxically, loss of POMC *Glp1r* expression increased HFD-induced weight gain, suggesting distinct roles for GLP-1R signaling in different hypothalamic regions. Elevated HFD-induced weight gain in GLP-1RKD$^{\Delta POMCcre}$ mice was not associated with significant changes in body composition, food intake or

EE. One potential explanation is that weight gain was measured in *ad lib* fed conditions, whereas body composition was measured in 5h-fasted mice. Thus, POMC GLP-1R may regulate the handling of nutrient stores during fasting. Unlike Nkx2.1 neuron- and POMC-specific *Glp1r* knockdown models, GLP-1RKD$^{\Delta Sim1cre}$ mice displayed no effects on body composition or energy balance parameters. These observations highlight the complexity of brain GLP-1R actions on the maintenance of energy balance and suggest that different hypothalamic nuclei mediate distinct effects of GLP-1, consistent with previous findings demonstrating nuclei-specific effects of GLP-1R signaling in visceral illness and HPA-axis activation (16).

Peripheral administration of GLP-1RA reduces food intake and body weight likely due, in part, to their ability to cross the blood brain barrier (17; 45). Indeed, disruption of pan-neuronal *Glp1r* expression inhibits the anorectic effects of peripherally-administered liraglutide (3). We hypothesized that this is due to loss of hypothalamic *Glp1r* expression. Surprisingly, the acute anorectic effects of peripherally-dosed Ex4 and liraglutide were preserved in our hypothalamic GLP-1R knockdown models. We then tested whether the hypothalamic GLP-1R mediates the long-term weight loss effects of GLP-1RA. Targeted delivery of the GLP-1R antagonist Ex9 to the ARC attenuates the weight loss effect of a 14-day liraglutide dosing regimen (17). In the present studies, chow-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice displayed similar weight loss as controls following a 14-day liraglutide treatment but were refractory to liraglutide-induced weight loss when fed a HFD. Importantly, in both the present studies and those by Secher *et al* (17), pharmacological or genetic blockade of the ARC or POMC GLP-1R did not prevent the initial weight loss induced by liraglutide. These findings implicate other hypothalamic regions and/or extrahypothalamic regions as mediators of the anorexigenic effects of GLP-1RA.

Modulation of the brain GLP-1R affects glucose production and utilization under hyperinsulinemic conditions (24; 46). We show that glucose tolerance is identical in GLP-

1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta Sim1cre}$ and GLP-1RKD$^{\Delta POMCcre}$ mice compared to controls. Moreover, Ex9 impaired glucose tolerance in GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice, suggesting that these GLP-1R populations do not mediate Ex9-induced impairments in glucose homeostasis. This is distinct from observations in β-cell GLP-1RKO mice in which Ex9 does not impair glucose tolerance (47). Liraglutide improved glucose tolerance in GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice to similar degrees as their respective controls. These observations were surprising considering that targeted delivery of Ex4 to the PVN and ARC improved glucose tolerance. Nevertheless, these results support observations by Sisley *et al* that loss of CNS GLP-1R expression does not affect glucose tolerance or the glucoregulatory effects of liraglutide.

Although the Cre lines in these studies primarily target hypothalamic neurons, there are interpretative caveats including incomplete Cre expression and off-target effects. We achieved only a ~50% reduction in *Glp1r* expression in GLP-1RKD$^{\Delta Nkx2.1cre}$ mice. One potential explanation is that Nkx2.1 is expressed in GABAergic but not glutamatergic neurons (48), raising the possibility that the GLP-1R may remain expressed in hypothalamic glutamatergic neurons. Sim1 is not only expressed in the PVN, but it is also enriched in the cerebellum, midbrain and hippocampus (21). Although enriched in the ARC, POMC is also expressed in the hippocampus and hindbrain (20). Moreover, there are non-POMC and non-NPY/AgRP ARC neurons that are GLP-1R-positive (14).

These studies demonstrate that GLP-1R expression within two hypothalamic regions typically assigned a prominent role in regulating energy balance, the ARC and PVN, is sufficient but not necessary for the effects of GLP-1RA on energy balance and highlight the importance of combining targeted pharmacological and genetic approaches to unravel the complex mechanisms by which GLP-1RA maintain nutrient homeostasis. The identification of overlapping CNS sites of action and signaling pathways for GLP-1R-mediated regulation of homeostatic and hedonic aspects of feeding behavior may inform the development of more effective therapies for T2D and obesity.

Acknowledgements

The authors would like to recognize John Shelley (SBP at Lake Nona) for his assistance with the RNA ISH studies.

Author Contributions: Melissa A. Burmeister and Julio E. Ayala designed the experiments. Melissa A. Burmeister, Hannah Smouse, Adriana Landivar-Rocha and Jennifer E. Ayala collected and analyzed the data. Daniel J. Drucker, Darleen A. Sandoval, Randy J. Seeley and Doris A. Stoffers provided essential research tools. Melissa A. Burmeister, Julio E. Ayala, Jacob D. Brown, Jennifer E. Ayala and Darleen A. Sandoval wrote the manuscript. All authors reviewed and edited the manuscript. Melissa A. Burmeister is the guarantor of this work and, as such, had full access to the all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analyses. This work was supported by NIH 4R01DK097361 to Julio E. Ayala; NIH 1R01DK107652 to Randy J. Seeley; and NIH 7R01DK082480 to Darleen A. Sandoval. Daniel J. Drucker is supported by the Canada Research Chairs program and CIHR grant 123391.

The authors report no conflict of interest. Randy J. Seeley has received research support from Ethicon Endo-Surgery, Novo Nordisk, Sanofi and Janssen. Randy J. Seeley has served on scientific advisory boards for Ethicon Endo-Surgery, Daiichi Sankyo, Janssen, Novartis, Nestle, Takeda, Boehringer-Ingelheim, Sanofi and Novo Nordisk. Randy J. Seeley is also a paid speaker for Ethicon Endo-Surgery. Darleen A. Sandoval has received research support from Ethicon Endo-Surgery, Novo Nordisk and Boehringer Ingelheim. Daniel J. Drucker has received consulting honoraria related to GLP-1 from MedImmune and Novo Nordisk, Inc.

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Figure legends

Figure 1. Direct injection of Ex4 into the PVN or ARC of the hypothalamus robustly suppresses food intake, whereas GLP-1 only modestly suppresses food intake. The values are mean±SEM and represent the time-course of cumulative 18-h food intake in chow-fed C57Bl/6J mice receiving GLP-1 or Ex4 (0.0025, 0.005 or 0.025µg) or artificial cerebrospinal fluid (ACSF, 100nL bilateral (PVN) or unilateral (ARC)) in the **(A and C)** PVN or **(B and D)** ARC. n=7-15 mice/group. *p<0.05 vs. ACSF.

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Figure 3. Chow diet-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice exhibit elevated food intake and energy expenditure compared to GLP-1R$^{f/f}$ controls. The values are mean±SEM and represent 5h-fasted body composition (body weight, lean mass and fat mass), cumulative 48-h food intake and 48-h energy expenditure in **(A, D and G)** GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=12), **(B, E and H)** GLP-1RKD$^{\Delta POMCcre}$ (n=10) and **(C, F and I)** GLP-1RKD$^{\Delta Sim1cre}$ (n=11) mice compared to GLP-1R$^{f/f}$ controls (n=12-15). *p<0.05 vs. GLP-1R$^{f/f}$.

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Figure 5. Disruption of the GLP-1R in Nkx2.1 neurons, POMC neurons or the PVN does not blunt the food intake-suppressive effect of peripherally-dosed Ex4. The values are mean±SEM and represent 16-h food intake following treatment with Ex4 (3µg, i.p.) in chow diet- or HFD-fed **(A and D)** GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=11-12), **(B and E)** GLP-1RKD$^{\Delta POMCcre}$ (n=7-8) and **(C and F)** GLP-1RKD$^{\Delta Sim1cre}$ (n=11) mice compared to GLP-1R$^{f/f}$ controls (n=10-15). Data are shown at 4-h intervals and expressed as a percentage of the food intake response observed following treatment with vehicle.

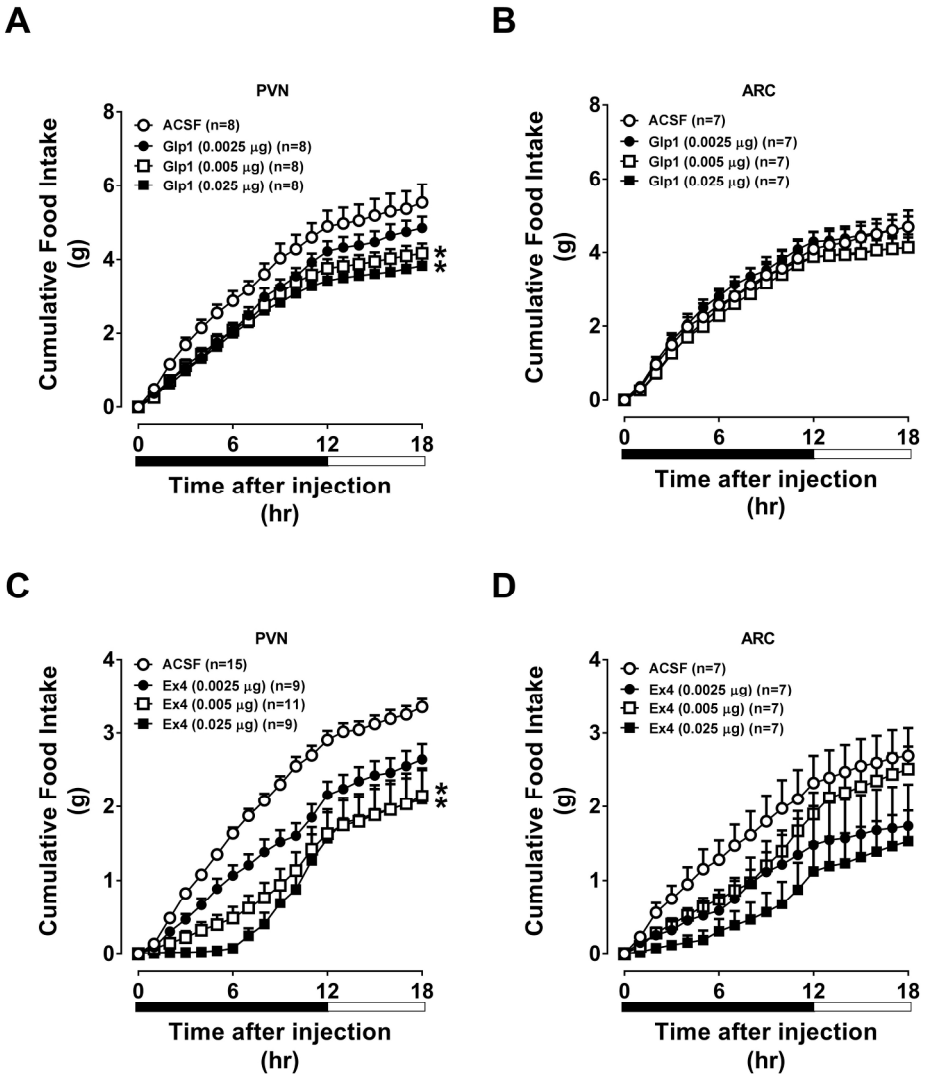
Figure 6. Disruption of the GLP-1R in Nkx2.1 neurons does not blunt the food intake-suppressive effect of peripherally-dosed liraglutide but does impact the compound's body-weight lowering effect in HFD-fed mice. **(A)** The values are mean±SEM and represent 16-h food intake following treatment with liraglutide (200µg, s.c.) in chow diet-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=9) mice compared to GLP-1R$^{f/f}$ controls (n=17). Data are shown at 4-h intervals and expressed as a percentage of the food intake response observed following treatment with vehicle. **(B and C)** The values are mean±SEM and represent daily body weight over the course of 21 days in **(B)** chow diet-fed or **(C)** HFD-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=5-6) compared to GLP-1R$^{f/f}$ controls (n=8-11) treated

with liraglutide. Data are expressed as a percentage of baseline (i.e., prior to liraglutide treatment) body weight. On days 0 through 13, morning body weight was measured, and animals received a twice-daily injection of liraglutide (200µg/kg BW, s.c.) or vehicle. On recovery days 14-21, only morning body weight was measured. *p<0.05 vs. saline. †p<0.05 vs. GLP-1R$^{f/f}$.

Figure 7. Direct injection of Ex4 into the PVN or ARC of the hypothalamus robustly improves glucose tolerance, whereas disruption of the hypothalamic GLP-1R in GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta POMCcre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice does not affect glucose tolerance. The values are mean±SEM and represent glucose excursion in chow-fed C57Bl/6J mice following a gavage of glucose (2g/kg BW, i.p.) and treatment with Ex4 (0.025µg) or ACSF (100nL) in the **(A)** PVN (n=8-9), **(B)** ARC (n=7-8) or **(C)** cortex (n=8-9) at time = 0 min. Inset: area under the curve (AUC) above baseline for each group. *p<0.05 vs. ACSF. The values are mean±SEM and represent glucose excursion in chow diet- or HFD-fed **(D, G and J)** GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=9-12), **(E, H and K)** GLP-1RKD$^{\Delta POMCcre}$ (n=7-10) and **(F, I and L)** GLP-1RKD$^{\Delta Sim1cre}$ (n=9-11) mice compared to GLP-1R$^{f/f}$ controls (n=10-15). **(D, E and F)** chow diet, OGTT; **(G, H and I)** chow diet, IPGTT; **(J, K and L)** HFD, OGTT. Inset: area under the curve (AUC) above baseline for each group. *p<0.05 vs. ACSF.

Figure 8. Disruption of the GLP-1R in GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice does not impact glucose tolerance following pre-treatment with Ex9 or liraglutide. The values are mean±SEM and represent glucose excursion in chow diet-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=7-10) and GLP-1RKD$^{\Delta Sim1cre}$ (n=7-12) pre-treated with **(A)** Ex9 (50µg, i.p.) vs. vehicle or **(B)** liraglutide (400µg/kg BW, s.c.) vs. vehicle 15-min (for Ex9 studies) or 120-min (for liraglutide studies) prior

to an i.p. glucose challenge. Inset: area under the curve (AUC) above baseline for each group. *$p<0.05$ vs. saline. $^{\dagger}p<0.05$ vs. GLP-$1R^{f/f}$.



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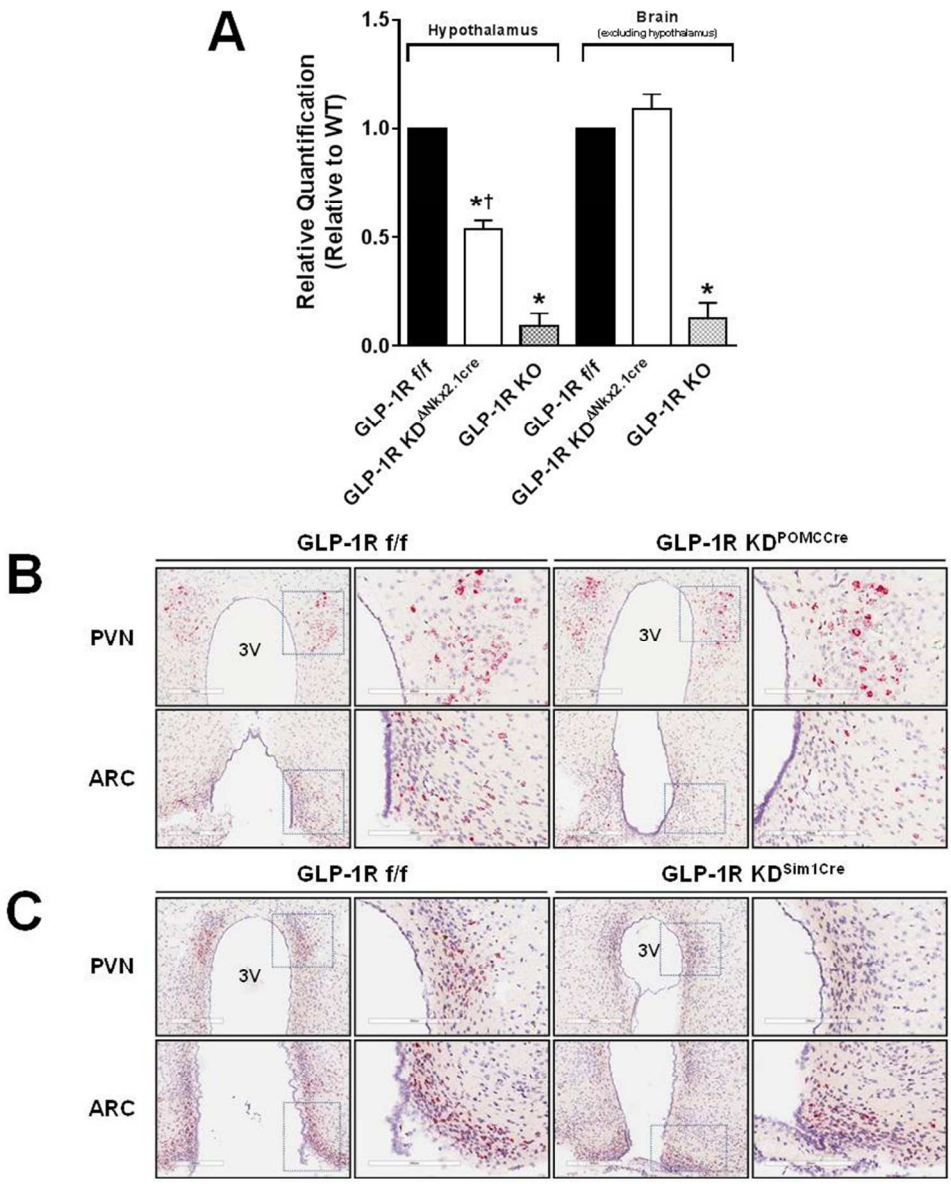

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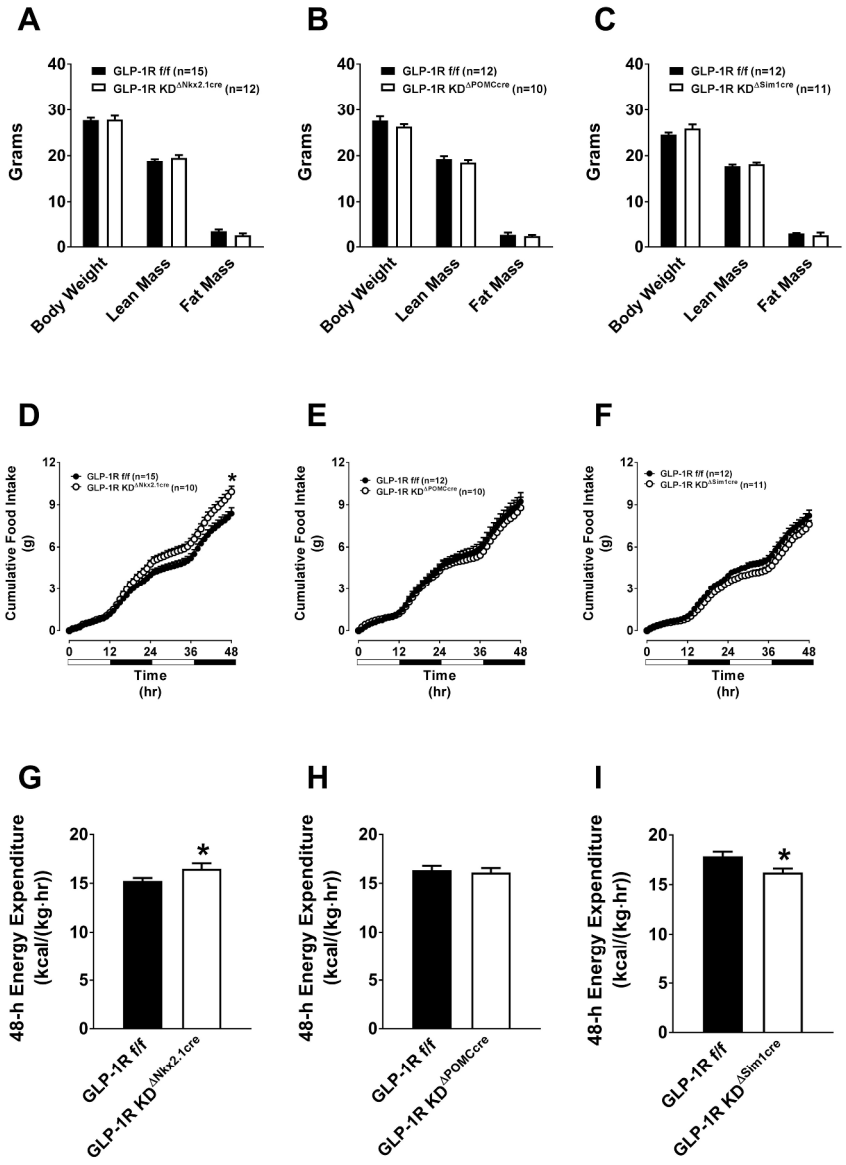

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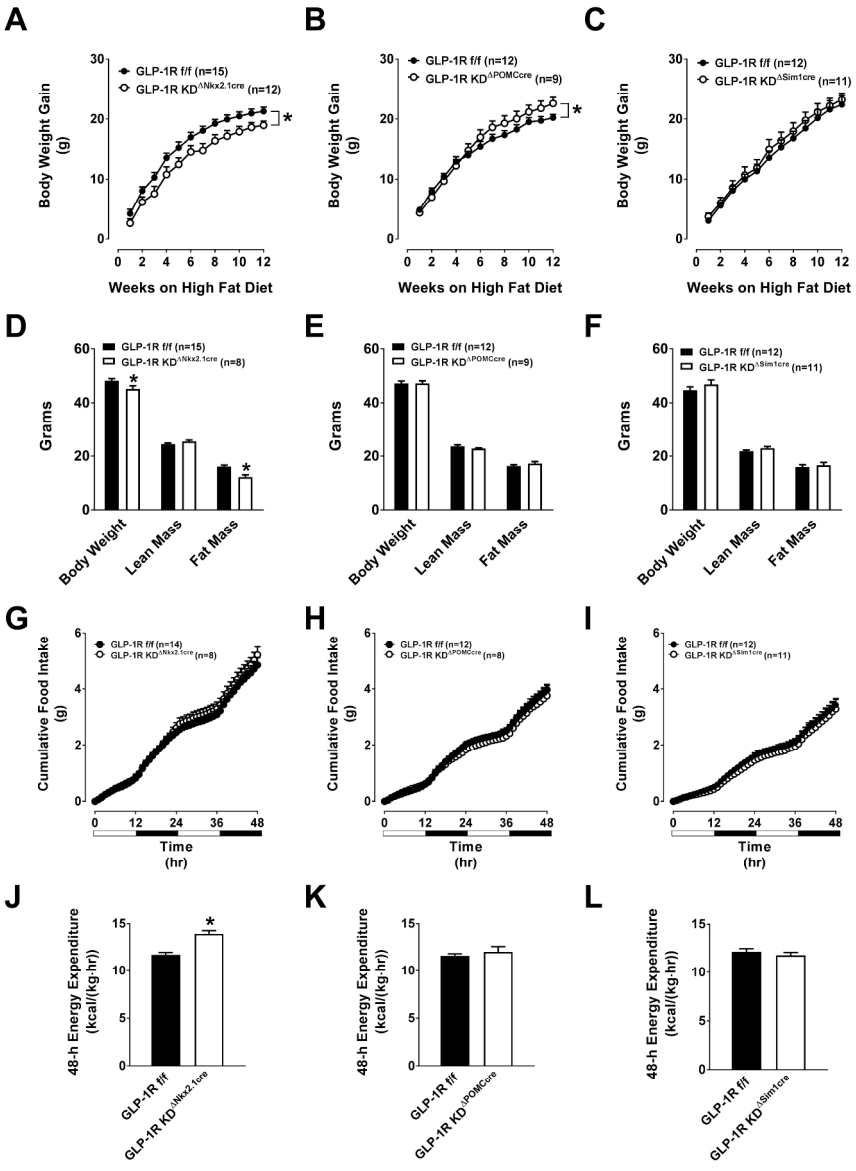

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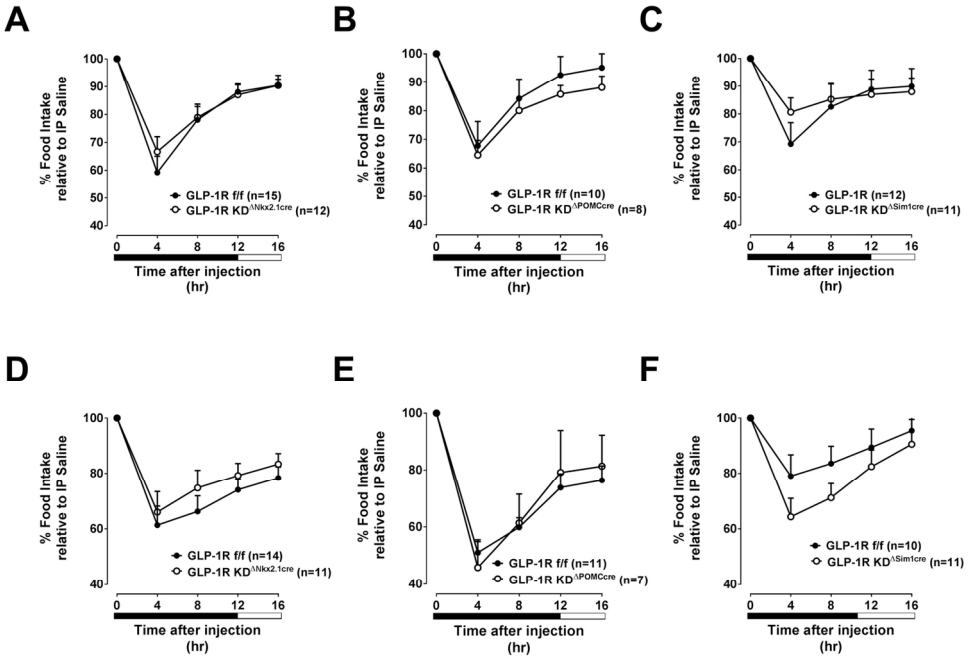

Figure 5. Disruption of the GLP-1R in Nkx2.1 neurons, POMC neurons or the PVN does not blunt the food intake-suppressive effect of peripherally-dosed Ex4. The values are mean±SEM and represent 16-h food intake following treatment with Ex4 (3µg, i.p.) in chow diet- or HFD-fed **(A and D)** GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=11-12), **(B and E)** GLP-1RKD$^{\Delta POMCcre}$ (n=7-8) and **(C and F)** GLP-1RKD$^{\Delta Sim1cre}$ (n=11) mice compared to GLP-1R$^{f/f}$ controls (n=10-15). Data are shown at 4-h intervals and expressed as a percentage of the food intake response observed following treatment with vehicle.

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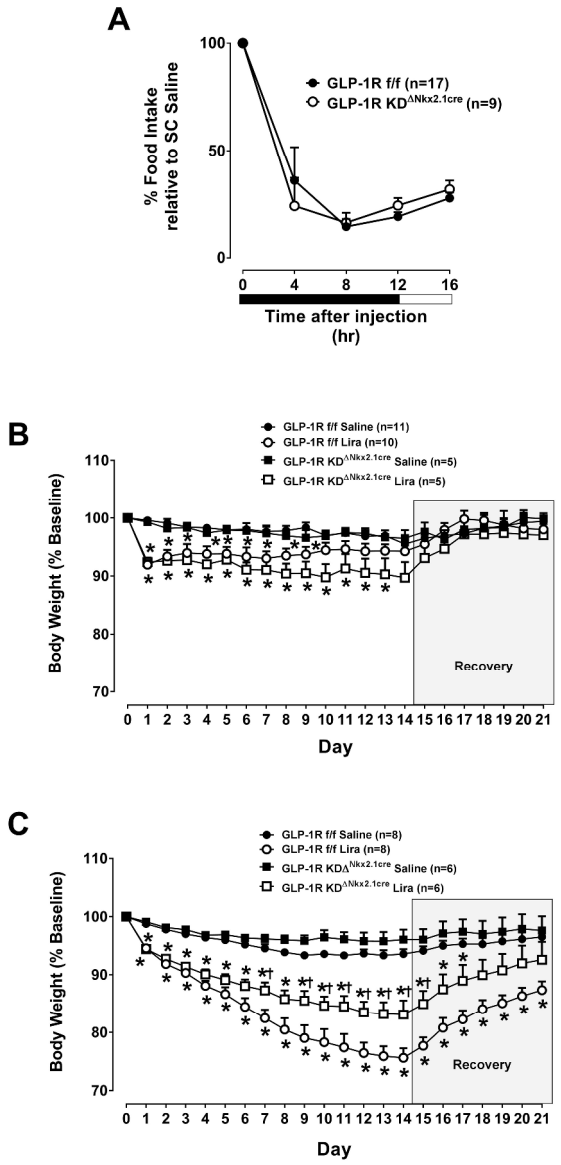

Figure 6. Disruption of the GLP-1R in Nkx2.1 neurons does not blunt the food intake-suppressive effect of peripherally-dosed liraglutide but does impact the compound's body-weight lowering effect in HFD-fed mice. (A) The values are mean±SEM and represent 16-h food intake following treatment with liraglutide (200µg, s.c.) in chow diet-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=9) mice compared to GLP-1R$^{f/f}$ controls (n=17). Data are shown at 4-h intervals and expressed as a percentage of the food intake response observed following treatment with vehicle. **(B and C)** The values are mean±SEM and represent daily body weight over the course of 21 days in **(B)** chow diet-fed or **(C)** HFD-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=5-6) compared to GLP-1R$^{f/f}$ controls (n=8-11) treated with liraglutide. Data are expressed as a percentage of baseline (i.e., prior to liraglutide treatment) body weight. On days 0 through 13, morning body weight was measured, and animals received a twice-daily injection of liraglutide (200µg/kg BW, s.c.) or vehicle. On recovery days 14-21, only morning body weight was measured. *p<0.05 vs. saline. †p<0.05 vs. GLP-1R$^{f/f}$.

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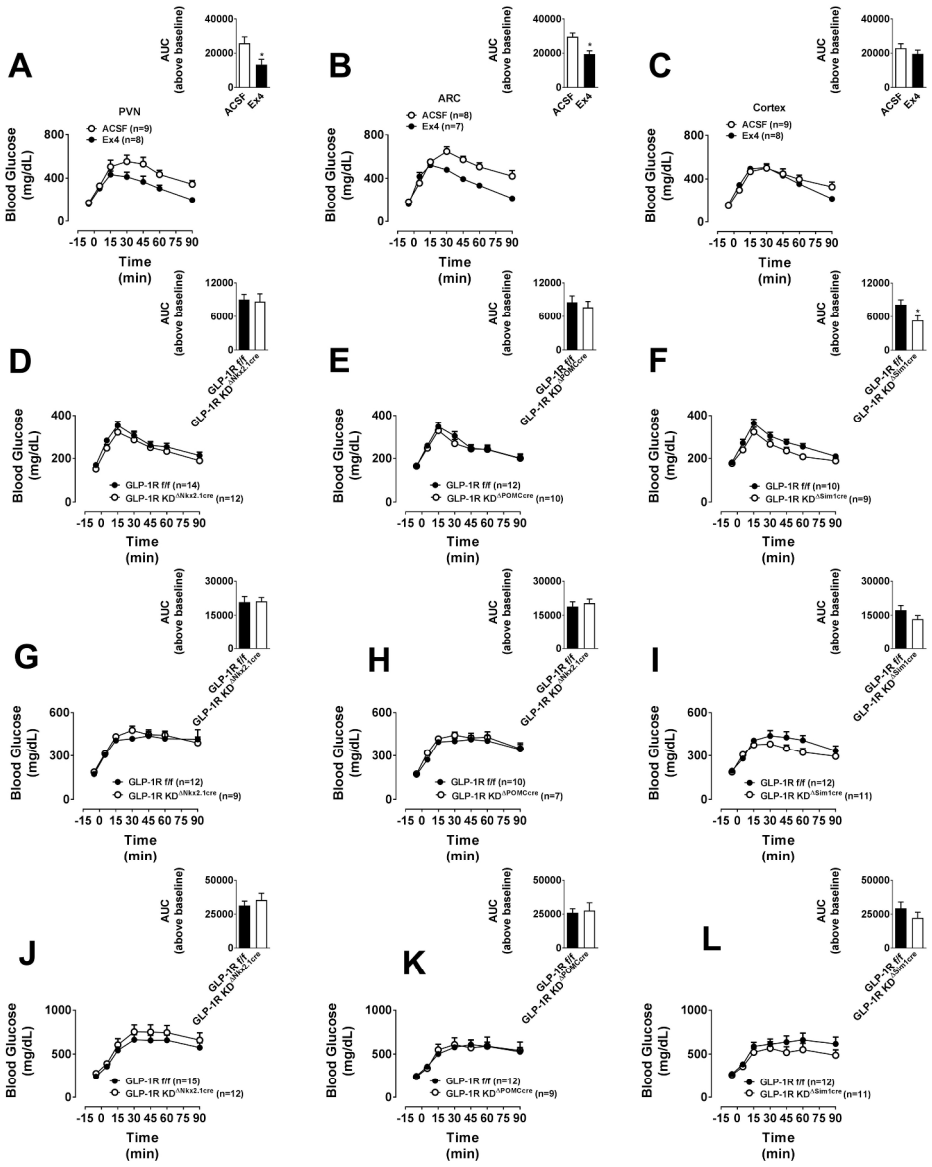

<u>Figure 7</u>. Direct injection of Ex4 into the PVN or ARC of the hypothalamus robustly improves glucose tolerance, whereas disruption of the hypothalamic GLP-1R in GLP-1RKD$^{\Delta Nkx2.1cre}$, GLP-1RKD$^{\Delta POMCcre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice does not affect glucose tolerance. The values are mean±SEM and represent glucose excursion in chow-fed C57Bl/6J mice following a gavage of glucose (2g/kg BW, i.p.) and treatment with Ex4 (0.025µg) or ACSF (100nL) in the **(A)** PVN (n=8-9), **(B)** ARC (n=7-8) or **(C)** cortex (n=8-9) at time = 0 min. Inset: area under the curve (AUC) above baseline for each group. *p<0.05 vs. ACSF. The values are mean±SEM and represent glucose excursion in chow diet- or HFD-fed **(D, G and J)** GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=9-12), **(E, H and K)** GLP-1RKD$^{\Delta POMCcre}$ (n=7-10) and **(F, I and L)** GLP-1RKD$^{\Delta Sim1cre}$ (n=9-11) mice compared to GLP-1R$^{f/f}$ controls (n=10-15). **(D, E and F)** chow diet, OGTT; **(G, H and I)** chow diet, IPGTT; **(J, K and L)** HFD, OGTT. Inset: area under the curve (AUC) above baseline for each group. *p<0.05 vs. ACSF.

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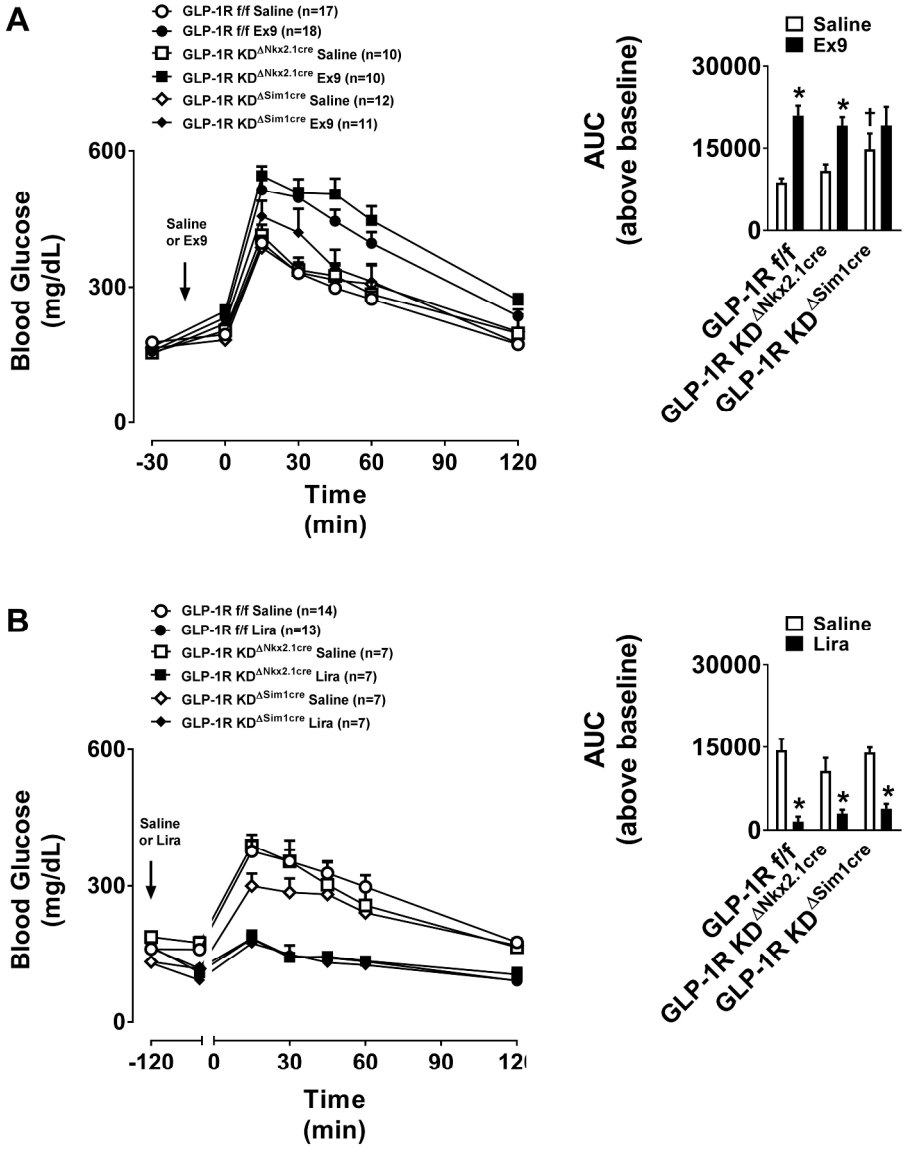

Figure 8. Disruption of the GLP-1R in GLP-1RKD$^{\Delta Nkx2.1cre}$ and GLP-1RKD$^{\Delta Sim1cre}$ mice does not impact glucose tolerance following pre-treatment with Ex9 or liraglutide. The values are mean±SEM and represent glucose excursion in chow diet-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=7-10) and GLP-1RKD$^{\Delta Sim1cre}$ (n=7-12) pre-treated with **(A)** Ex9 (50µg, i.p.) vs. vehicle or **(B)** liraglutide (400µg/kg BW, s.c.) vs. vehicle 15-min (for Ex9 studies) or 120-min (for liraglutide studies) prior to an i.p. glucose challenge. Inset: area under the curve (AUC) above baseline for each group. *p<0.05 vs. saline. †p<0.05 vs. GLP-1R$^{f/f}$.

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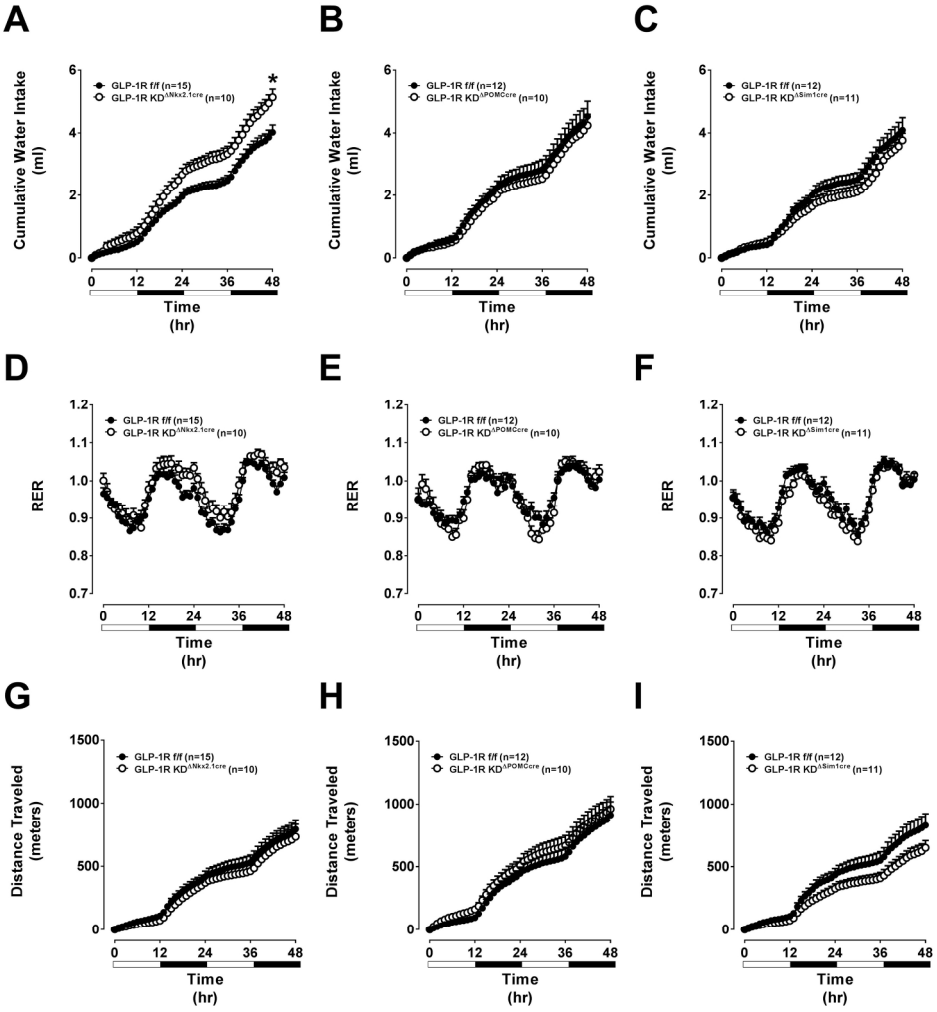
Supplemental Table 1. Analysis of covariance (ANCOVA) for fat mass and 48-h energy expenditure in GLP-1RKD$^{\Delta Nkx2.1cre}$ vs. GLP-1R$^{f/f}$ mice. ANCOVA was performed using the Mouse Metabolic Phenotyping Center (MMPC) web-based Multiple Linear Regression program (http://www.mmpc.org/shared/regression.aspx). The response variables were fat mass and average 48-h energy expenditure, and the covariates were total body mass and average 48-h food intake. Analyses were performed for chow diet and HFD studies where relevant. Data are presented as mean±SEM for n=8-15 mice per group.

Supplemental Figure 1. Chow diet-fed GLP-1RKD$^{\Delta Nkx2.1cre}$ mice exhibit elevated water intake but normal RER and locomotor activity compared to GLP-1R$^{f/f}$ controls. The values are mean±SEM and represent cumulative 48-h water intake, RER and locomotor activity in chow diet-fed **(A, D and G)** GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=10), **(B, E and H)** GLP-1RKD$^{\Delta POMCcre}$ (n=10) and **(C, F and I)** GLP-1RKD$^{\Delta Sim1cre}$ (n=11) mice compared to GLP-1R$^{f/f}$ controls (n=12-15). Locomotor activity is expressed as cumulative distance traveled. *$p<0.05$ vs. GLP-1R$^{f/f}$.

Supplemental Figure 2. HFD-fed GLP-1R KD$^{\Delta Nkx2.1cre}$ mice exhibit elevated water intake but normal RER and locomotor compared to GLP-1R$^{f/f}$ controls. The values are mean±SEM and represent cumulative 48-h water intake, RER and locomotor activity in HFD-fed **(A, D and G)** GLP-1RKD$^{\Delta Nkx2.1cre}$ (n=11), **(B, E and H)** GLP-1RKD$^{\Delta POMCcre}$ (n=8) and **(C, F and I)** GLP-1RKD$^{\Delta Sim1cre}$ (n=11) mice compared to GLP-1R$^{f/f}$ controls (n=12-14). Locomotor activity is expressed as cumulative distance traveled. *$p<0.05$ vs. GLP-1R$^{f/f}$.

Analysis of covariance (ANCOVA)			
Chow Diet			
Response Variable = Fat Mass Covariate = Total Body Mass			
	GLP-1R f/f	**GLP-1R KD$^{\Delta Nkx2.1cre}$**	**p-value**
Overall Mean	3.44±0.314	2.54±0.351	0.06642
Group Means	3.43±0.314	2.56±0.351	0.07735
Response Variable = 48-h Energy Expenditure Covariate = 48-h Food Intake			
	GLP-1R f/f	**GLP-1R KD$^{\Delta Nkx2.1cre}$**	**p-value**
Overall Mean	7.20±0.201	7.81±0.252	0.0391
Group Means	7.05±0.191	7.54±0.234	0.0162
Response Variable = 48-h Energy Expenditure Covariate = Total Body Mass			
	GLP-1R f/f	**GLP-1R KD$^{\Delta Nkx2.1cre}$**	**p-value**
Overall Mean	7.00±0.173	7.61±0.212	0.03721
Group Means	7.05±0.173	7.54±0.211	0.04399
High Fat Diet			
Response Variable = 48-h Energy Expenditure Covariate = Total Body Mass			
	GLP-1R f/f	**GLP-1R KD$^{\Delta Nkx2.1cre}$**	**p-value**
Overall Mean	9.25±0.213	10.56±0.242	0.0007376
Group Means	9.38±0.205	10.38±0.232	0.0039170



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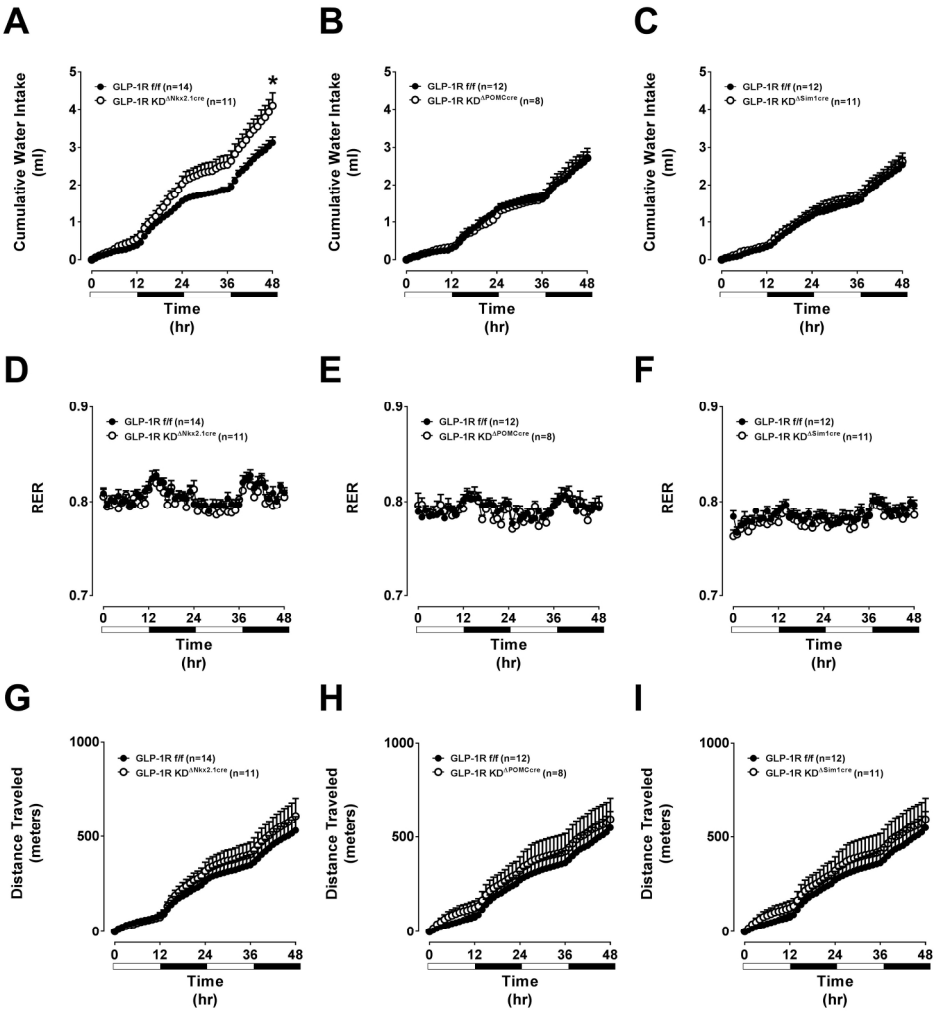

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